ACTA PHYSIOLOGICA SCANDINAVICA
VOL. 38 . SUPPLEMENTUM 130

MECHANISM OF ACTIVATION AND EFFECT OF PLASMIN IN BLOOD

BY
STEN MÜLLERTZ

UNIVERSITY OF MICHIGAN
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1956

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FROM THE BIOLOGICAL INSTITUTE
OF THE CARLSBERG FOUNDATION, COPENHAGEN,
DENMARK

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1956

Denne afhandling er i forbindelse med omstående tidligere publicerede afhandlinger af det lægevidenskabelige fakultet ved Københavns universitet antaget til offentlig at forsvares for den medicinske doktorgrad.

København, den 1. juni 1956.

P. Bonnevie
h. a. dec.

NORDLUNDES BOGTRYKKERI, KØBENHAVN

PREVIOUSLY PUBLISHED PAPERS

The Estimation of Plasmin and Trypsin by Means of the Plate Method. Müllertz, S.: Acta Physiol. Scand. 1952, *25*, 93.

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Formation and Properties of the Activator of Plasminogen and of Human and Bovine Plasmin. Müllertz, S.: Biochem. J. 1955, *61*, 424.

TO MY PARENTS

PREFACE

This monograph is a review of experimental studies on the proteolytic enzyme of blood. My own experimental studies have been performed during may stay as a research associate at the Biological Institute of the Carlsberg Foundation, Copenhagen, during the years 1952-1955. Thanks are due to ALBERT FISCHER, M.D., Director of the institute, for his kind interest and excellent working facilities.

The studies on the proteolytic enzyme of blood at the institute were performed by a team of research workers under the leadership of TAGE ASTRUP, D.Sc. I wish to express my sincere gratitude for the friendly and stimulating interest with which he followed the work. His advice and generous support as well as the excellent relations prevailing in the department have been invaluable.

I wish to express my warm appreciation to my friend and collaborator, MOGENS LASSEN M.Sc., for his continued interest, helpfulness and valuable suggestions presented in numerous discussions on mutual problems.

I am deeply grateful to Miss LENE FELDINGER for her skilful technical assistance and generous help.

I am greatly indebted to Dr. ROGER HARDISTY for reading through my papers and to Dr. AUDREY FJELDE for reading through this review. Their amendments and suggestions concerning the English style and vocabulary were a great help.

The investigations were financed by grants from the JOSIAH MACY JR. FOUNDATION, New York, and by THE NATIONAL DANISH ASSOCIATION AGAINST RHEUMATIC DISEASES to Dr. TAGE ASTRUP. Thanks are due to LØVENS KEMISKE FABRIK for generous supply of purified thrombin and LEDERLE LABORATORIES DIVISION for ample supply of streptokinase ("Varidase").

This review with previously published papers was submitted to the Medical Faculty of the University of Copenhagen 26 June 1955 and accepted as a doctorate thesis 1 June 1956.

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INTRODUCTION

Blood contains an inactive precursor of a proteolytic enzyme, which is converted into active enzyme by different substances in vitro and under various conditions in vivo. In the course of previous work on this enzyme system different terms have been applied to the components involved. A discussion of the terminology has been published by FERGUSON (1948). Such terms as plasma trypsin (SCHMITZ 1936) and serum tryptase (FERGUSON 1943) are unsuitable, because they imply a relationship between the enzyme in blood and trypsin. At present only two terms are commonly used. LOOMIS, GEORGE and RYDER (1947) proposed the term fibrinolysin to the active enzyme, profibrinolysin to the precursor, and antifibrinolysin to the inhibitor in blood. This terminology appears undesirable, since the enzyme is not specific for fibrin, but is a general proteolytic enzyme capable of splitting a certain type of peptide linkage. CHRISTENSEN and MACLEOD (1945) suggested the term plasmin for the enzyme, in conformity with common usage for proteases, where the prefix indicates the source of the enzyme. The precursor was termed plasminogen, the plasmin inhibitor in blood, antiplasmin. The streptococcal factor, previously called fibrinolysin, is now exclusively termed streptokinase, as it is no fibrinolytic enzyme proper, but an agent which effects the transformation of the precursor into active enzyme. In this review, the nomenclature of CHRISTENSEN and MACLEOD is generally adhered to.

This review is concerned primarily with the mechanism of activation of plasminogen and the effect of plasmin in blood. The different ways, in which the transformation of plasminogen to plasmin takes place, are discussed and a description is given of the properties and mechanism of interaction of components in blood and other substances, which are involved in these processes.

Recent developments have shown that the results are not identical when serum, plasma or different globulin preparations are used, because of different proportions between the components involved in the reac-

tions. For the same reason it is also of great significance whether material from blood of man or from different animals is used. Furthermore, results obtained with substrates of clotted fibrinogen, which contain considerable amounts of plasminogen, yield information primarily about activators of plasminogen. Plasmin can be assayed properly only by means of protein substrates free from plasminogen. Therefore, whenever possible, detailed information is given about the mode of preparation and source of the material and about the substrate used for the assay.

Some subjects, which are not strictly relevant, have not been thoroughly reviewed. Among these the significance of the inhibitors in blood has recently been studied and reviewed by JACOBSSON (1955). The discrepant views concerning the role of plasmin in blood coagulation have been discussed by NOLF (1938), TRAVIS and FERGUSON (1951) and by BIGGS and MACFARLANE (1953). Those concerning the relation of proteolysis in blood to anaphylaxis in vivo and in vitro have been reviewed by UNGAR (1952). Brief reference is made in the text to clinical observations on moderate and excessive fibrinolysis, while other clinical studies and applications have not been discussed. Reviews covering most problems related to the proteolytic enzyme of blood have been published by MACFARLANE and BIGGS (1948), HALSE (1948), PERMIN (1949), and by BIGGS and MACFARLANE (1953).

ACTIVATION OF PLASMINOGEN

1. Development of fibrinolytic activity in the organism

Many observations have shown that proteolytic and fibrinolytic activity may be present in globulin fractions prepared in different ways from normal blood. Thus, CLIFFTON and DOWNIE (1950) and CLIFFTON and CANNAMELA (1951, 1953a) observed a considerable proteolytic and fibrinolytic activity in an euglobulin fraction from normal dog, rabbit, and guinea pig blood, while the spontaneous activity of fractions from man and some other animals was weak. MACFARLANE and colleagues have especially contributed to the knowledge of the development of fibrinolytic activity in human blood. MACFARLANE and PILLING (1946) added human fibrinogen and thrombin to serial dilutions of plasma or of the euglobulin fraction from human blood, and they determined the lytic activity from the highest dilution which caused lysis of the clots in 24 hours at 37°C. Normal plasma showed no lysis, while normal globulin showed a moderate lytic activity, which could be increased considerably by different means. Active plasma was obtained from human individuals subjected to various forms of stress known to induce increased fibrinolysis. This plasma showed no lysis undiluted, but a moderate activity after appropriate dilution. Active globulin from such plasma samples showed a stronger lytic activity than normal globulin, but the activity was lost when the globulin was recombined with pseudoglobulin-albumin in the normal proportions. From these observations it was concluded that normal blood contains small and varying amounts of a proteolytic enzyme, completely neutralized in blood by an inhibitor. The effect of the inhibitor is decreased by precipitation of the globulin fraction or by dilution of plasma. Clots of diluted plasma showed no lysis in 24 hours at 37°, when obtained from normal individuals, but were a sensitive indicator of the development of an increased fibrinolytic activity in vivo. Using this technique, FEARNLEY, REVILL and TWEED (1952) reported on the lability of the active substance in these samples and observed lysis of clots prepared also from

normal human individuals, when precautions were taken against deterioration (Fearnley and Tweed 1953). A small or moderate increase of the fibrinolytic activity of human blood has been observed to develop in various conditions of physical and emotional stress. A review of these observations is given by Macfarlane and Biggs (1948), Halse (1948) and Biggs and Macfarlane (1953). In these cases the fibrinolytic activity was slight and no effects in the organism were perceptible. However, in certain pathological states fibrinolytic activities develop, which are sufficient to destroy all fibrin and fibrinogen in the blood in a very short time. Nolf (1905) in his early experiments observed progressive fibrinolysis and fibrinogenopenia after hepatectomy and injection of peptone in dogs. Tagnon, Levenson, Davidson and Taylor (1946) observed a complete lysis of whole blood clots within 3 hours at 37°C in patients suffering from severe burns and from haemorrhagic shock, and similar observations were made in experimentally induced haemorrhagic shock in dogs. Since these early studies, numerous reports have appeared on the development of excessive fibrinolysis with fibrinogenopenia or with afibrinogenaemia, uncoagulable blood, a marked haemorrhagic diathesis and a serious often fatal course in different pathological states. Such cases have been studied, and relevant literature has been reviewed by Bennike and Müllertz (1952), Ratnoff (1952 b) and Reid, Weiner and Roby (1953).

The state of blood after death has been an object of much study for many years especially from a medico-legal point of view. An extensive survey of these studies was presented by Berg (1950). In some cases the blood clots more or less slowly and remains clotted for many days, in other cases the clotting is slow and partial, and the clots are dissolved after some time. In a number of cases the blood shows no clotting but remains fluid and incoagulable, containing no fibrinogen or fibrin (Mole 1948, Berg 1950). Morawitz (1906) originally demonstrated the fibrinogenolytic and fibrinolytic properties of post-mortem fluid blood. Mole (1948) observed the presence of an active lysin in 90% of post-mortem cases, in which the blood was fluid, and similar observations were reported by Berg (1950). Mole (1948) considered the occurrence of fibrinolytic activity in blood after death to be the normal response to death of the healthy organism. Müllertz (1952 c) found no fibrinolytic activity, when death was preceded by cancer or infections, but on the other hand a rather high proportion of inactive blood was found in cases with no known history of illness before death and without any signs of preceding disease being demonstrable at autopsy. The suddenness of death has generally been considered an important factor in the develop-

ment of fibrinolytic activity post-mortem, but inactive blood was sometimes observed after traumatic instantaneous death (LENGGENHAGER 1938, MOLE 1948, MÜLLERTZ 1952 c). Death combined with acute anoxia has generally been associated with a very high fibrinolytic activity, but exceptions to this rule may occur (OKI 1934, BERG 1950, MÜLLERTZ 1952 c).

Many observations support the view that the development of fibrinolytic activity in blood in the organism is brought about by an interaction between substances in tissue and in blood. A physiological activator of plasminogen in tissue was originally demonstrated by ASTRUP and colleagues (ASTRUP and PERMIN 1947, and subsequent papers). HALSE (1948) observed a simultaneous increase in the phosphatide content and fibrinolytic activity of blood in the living human organism and after death in asphyxiated animals. He suggested that release of phosphatide-containing substances from tissue into blood was responsible for the production of increased fibrinolysis through a neutralization of inhibitory substances. MOLE (1948) found the lytic activity in post-mortem blood to be high in small vessels and relatively low in large vessels and in the heart, and he suggested that the development of fibrinolytic activity was related to the area of the endothelial surface. The strong lytic activity occurring after anoxaemic death indicated the involvement of an increased permeability of the endothelium followed by a release of activating substances into blood (OKI 1934, MOLE 1948, MÜLLERTZ 1952 c). Active post-mortem blood contains no fibrin or fibrinogen. In states of excessive fibrinolysis in the living human organism afibrinogenaemia and a haemorrhagic diathesis develop. In these cases defibrination of blood has probably to some extent been caused by the release of thromboplastic material from tissue into blood followed by intravascular clotting and a subsequent lysis of the clots. These findings are closely parallel to the effects produced in vivo by intravenous injection of thromboplastin. In animal experiments injection of large quantities of tissue extracts resulted in instantaneous death caused by universal intravascular clotting, while successive injections of subletal doses resulted in afibrinogenaemia and uncoagulable blood, no clots being demonstrable by autopsy (MILLS 1921, RATNOFF and CONLEY 1951). These observations may be compared with the effect of intravenous injection in dogs of dog plasmin and of staphylokinase (LEWIS and FERGUSON 1949). Staphylokinase caused the appearance of a strong fibrinolytic activity in blood accompanied by a fall in plasminogen and antiplasmin. The lysis times of whole blood clots decreased rapidly until no clots were formed on the addition of thrombin, indicating the com-

plete absence of fibrinogen caused by in vivo fibrinogenolysis. Similar results were observed after injection of plasmin, and gross post-mortem examination showed the presence of extensive haemorrhages in the tissues.

2. Activation of plasminogen by fractionation and by chloroform

Under certain conditions proteolytic and fibrinolytic activity develops in stored solutions of blood globulin. The active globulin fractions studied, have been prepared by ammonium sulphate precipitation from ox serum (HEDIN 1904, ASTRUP 1951 a), by precipitation at about pH 5.3 and low ionic strength from ox serum (HEDIN 1904, LUNDBLAD 1950), horse serum (ROSENMANN 1936), human serum (CHRISTENSEN 1945, MACFARLANE and PILLING 1946, RATNOFF 1948 c, 1952 a), dog serum (LEWIS and FERGUSON 1951 a) and from monkey, dog, rabbit and guinea pig serum (CLIFFTON and DOWNIE 1950, CLIFFTON and CANNAMELA 1951, 1953 a), by precipitation with trichloracetic acid and acetone from horse and human serum (SCHMITZ 1937) or by a low salt, low temperature, ethanol separation applied to human plasma (TAYLOR, DAVIDSON, TAGNON, ADAMS, MACDONALD and MINOT 1945, SHINOWARA 1947, ONCLEY, MELIN, RICHERT, CAMERON and GROSS Jr. 1949). A highly fibrinolytic fraction was prepared from pig, horse and ox serum by serial isoelectric and acetone precipitations and acid extractions (v. KAULLA 1949). The blood protease adheres to fibrin, and spontaneously lysed fibrin or fibrin eluates prepared from human and equine plasma (NOLF 1908, ROSENMANN 1920, 1922, 1936, 1937, SCHMITZ 1936) have been used as a source of the enzyme. By the ethanol fractionation of human plasma (ONCLEY et al 1949), the active enzyme was obtained after spontaneous lysis of a clot, which had formed spontaneously in a solution of fraction III-2,3 (COHN et al 1946). Casein (HEDIN 1904, SHINOWARA 1947, TAYLOR et al 1945, DOWNIE and CLIFFTON 1949, CLIFFTON and DOWNIE 1950, CLIFFTON and CANNAMELA 1951, 1953 a), gelatin (HEDIN 1904, CHRISTENSEN 1947 a, LUNDBLAD 1949, v. KAULLA 1949) and heat-coagulated serum (HEDIN 1904) were split by the different proteolytic preparations, while in other studies the lysis of fibrin was used to determine the activity of the preparations. Considerable spontaneous proteolytic activity has been observed in globulin solutions from certain animal sera (CLIFFTON and DOWNIE 1950, CLIFFTON and CANNAMELA 1951, 1953 a). In dog globulin (LEWIS and FERGUSON 1951 a), and in bovine globulin (ASTRUP

1951 a) a high fibrinolytic activity developed when the solutions were stored at 5°C for a few days to several months. However, in most cases, the fibrinolytic and proteolytic activity has been very slight, requiring long incubation periods to obtain significant effects on the substrates.

A considerable increase in proteolytic activity may be produced by a chloroform treatment of plasma, serum or globulin fractions. A large number of studies have been published on the activation of the blood protease by means of chloroform. Plasma from man (CHRISTENSEN and MACLEOD 1945) dog (NOLF 1921 b, 1922, TAGNON 1942) swine (HOUGARDY 1933, 1934), birds (NOLF 1921 a) and from man, dog, ox, rabbit and swine (TAGNON, DAVIDSON and TAYLOR 1942) has been used. By the procedure generally used, plasma was shaken with about 1/10 volume of chloroform and left stoppered at room temperature. Chloroform induced clotting of fibrinogen in the plasma, and the preparation was stored until the clot had lysed completely. A large amount of denatured protein was removed and a further purification of the protease was obtained by precipitating a globulin fraction from the supernatant (TAGNON, DAVIDSON and TAYLOR 1942, CHRISTENSEN and MACLEOD 1945). The lysis of the clot was found to proceed at a higher rate, when removed from the serum and washed with saline (HOUGARDY 1933, 1934). The presence of a fibrin clot is not necessary for the activation of the protease. Thus, serum was used in the original and in later studies (DENYS and MARBAIX 1889, DELEZENNE and POZERSKI 1903, YAMAKAWA 1918, CHRISTENSEN 1947 a). Furthermore, globulin solutions have been used, which have been prepared by ammonium sulphate precipitation or by precipitation at pH 5.3 and low ionic strength from human serum or plasma (KAPLAN 1944, ROCHA e SILVA and RIMINGTON 1948, RATNOFF 1948 b, PERMIN 1949), from dog serum (LEWIS and FERGUSON 1951 b) and from ox serum (LOOMIS, GEORGE and RYDER 1947, ROCHA e SILVA and RIMINGTON 1948, PERMIN 1949). Reproducible results were not always obtainable. Thus CHRISTENSEN and MACLEOD (1945) stated that fairly consistent results were obtained with small lots of human plasma, while inconsistent results and low yields were obtained with large quantities of plasma. PERMIN (1949) obtained appreciable fibrinolytic activity only in 10-20% of the activation experiments with bovine globulin solutions.

Protease activation has also been produced by treatment of serum or globulin solutions with ether, ethanol and thymol (DENYS and MARBAIX 1889) acetone, ethanol and other alcohols (YAMAKAWA 1918, STEPHAN and WOHL 1921, KAPLAN 1944) with certain oxidizing agents (LUNDBLAD 1950) and with urea (JÜHLING and WÖHLISCH 1938). These

observations should be considered in the evaluation of the comparatively high spontaneous activity occurring in globulin fractions prepared by acetone or ethanol fractionation techniques. HALSE (1948, 1951) reported that the spontaneous lysis of clots in diluted human plasma was increased after addition of heparin, lecithin and a number of adsorptive substances as kaolin and charcoal and suggested that the effect was due to a neutralization of inhibitory substances in blood. ASTRUP, CROOKSTON and MACINTYRE (1950) and ASTRUP and ALKJÆRSIG (1952 b) observed a strong inhibition by heparin of the lysis produced in a bovine fibrin substrate by bovine plasmin prepared by tissue activator and by human streptokinase-activated globulin. UNGAR and MIST (1949), GEIGER (1952 a) and UNGAR, DAMGAARD and HUMMEL (1953) described the release of fibrinolytic activity by addition of specific antigen to blood from previously sensitized guinea pigs. A similar activity was produced in normal guinea pig serum by addition of agar, peptone, heparin and some other substances anaphylactoid to this serum. Protamin has been reported to increase the fibrinolytic activity of dog blood (SCROGGIE, JAQUES and ROCHA e SILVA 1947) and the proteolytic activity of human and monkey globulin (DOWNIE and CLIFFTON 1949, CLIFFTON and DOWNIE 1950). No such effect was observed by FANTL and EVERARD (1950). Evidence against any participation of the blood protease in the mechanism of anaphylaxis in vivo and in vitro in rabbits was presented by MCINTIRE, ROTH and SPROULL (1950, 1952).

Different explanations have been proposed for the spontaneous or chloroform-induced development of proteolytic activity in serum globulin solutions. The protease present in such solutions was found to be counteracted by an inhibitor associated with the albumin-pseudoglobulin fraction (JACOBSSON 1955 and references). The serum protease was assumed normally to be bound by this inhibitor, while the function of chloroform was to free the protease from it (DELEZENNE and POZERSKI 1903, YAMAKAWA 1918).

CHRISTENSEN (1947 a) showed that the protease inhibitor was destroyed completely by chloroform. SCHMITZ (1937) prepared a protease and a protease inhibitor from human, horse, and ox blood and suggested the presence in blood of an enzyme-inhibitor complex, which was analogous to the dissociable trypsin-antitrypsin compound studied by HUSSAY and NORTHROP (1923). Dissociation of the hypothetical complex and liberation of enzyme were assumed to occur by precipitation of the globulin fraction, by adsorption onto fibrin or by a chloroform treatment resulting in destruction of the inhibitor (SCHMITZ 1937, MACFARLANE and PILLING 1946).

The evolvement of proteolytic activity after chloroform activation was studied in human serum (Christensen 1947 a), in human plasma globulin (Ratnoff 1948 b) and in ox globulin (Rocha e Silva and Rimington 1948). The results were to some extent in line with an autocatalytic activation. Chloroform was assumed to destroy inhibitor and to set free small amounts of enzyme, which initiated the autocatalytic reaction (Christensen and MacLeod 1945). On the other hand, Grob (1949) observed no appreciable inhibitory activity against serum protease and trypsin in human globulin fractions (fraction II and III, Cohn et al 1946), which developed slight spontaneous and strong chloroform-induced activities. He concluded that the formation of proteolytic activity by chloroform could not depend on a destruction of an inhibitor, but that chloroform in some way influenced the activation of plasminogen. A similar explanation was proposed by Permin (1949). A direct effect of chloroform on plasminogen was less probable. In some purified globulin preparations from bovine serum (Permin 1949), and from dog serum (Lewis and Ferguson 1951 b) no proteolytic activity was provoked by chloroform, even if the inhibitor content was very low and considerable quantities of plasminogen could be demonstrated. Finally, the effect of chloroform might consist in a destruction of substances, which were inhibitory to the activation of plasminogen. Schmitz (1936) was able to prepare two fractions from horse fibrin, both slightly proteolytic, which developed increased proteolytic activity after mixing. He suggested that a protease, its precursor and a kinase were present in blood, and that both kinase and protease were bound by an inhibitor. The existence of an antiplasminogen in bovine globulin activated by chloroform was also suggested by Hultin and Lundblad (1949). The effect of chloroform was identical in the presence and absence of calcium and in platelet-rich and platelet-poor plasma (Tagnon, Davidson and Taylor 1942, Lewis and Ferguson 1951 b), and a release of a kinase from cellular elements of blood by chloroform was therefore improbable. Lewis and Ferguson (1951 b) prepared a globulin fraction by ethanol precipitations from normal dog serum, which induced a strong fibrinolytic activity in a dog globulin fraction, during storage for several weeks at 5°. They suggested that the spontaneous activation and activation by chloroform were caused by a kinase, normally present in blood, and that the reaction was inhibited by an antilysin and possibly also by an antikinase in blood.

3. Activation of plasminogen by proteases

The development of proteolytic activity in serum or globulin solutions after chloroform treatment has been ascribed to an autocatalytic reaction. This assumption was based on the shape of the curve obtained by plotting proteolytic activity against incubation time (Christensen 1947 a, Rocha e Silva and Rimington 1948, Ratnoff 1948 b). An analysis of this reaction must be very uncertain, because of the limited knowledge of the components involved. Furthermore, Christensen and MacLeod (1945) found that the conversion of plasminogen to plasmin by streptokinase followed a first order reaction, indicating an enzymic effect of the kinase, but this observation is hardly conformable with a simultaneous autocatalytic activation of plasminogen. The problem has been investigated in a more direct way. Plasmin and plasminogen from ox blood (Astrup 1951 a) and from dog blood (Lewis and Ferguson 1952 a) could be kept in the same solution without any evidence of interaction, and bovine plasmin, activated spontaneously or by tissue activator, showed no increase in activity on addition of increasing amounts of bovine plasminogen (Astrup and Sterndorff 1952 a). In all instances the plasminogen could be activated by other means. On the other hand, small increases in activity were observed in mixtures of bovine chloroform-activated plasmin and human globulin (Kocholaty, Ellis and Jensen 1952) and bovine globulin (Jackson and Mertz 1954). However, activators of plasminogen have been found in horse globulin (Schmitz 1936), in dog globulin (Lewis and Ferguson 1951 b) and in human globulin (Müllertz 1953 b); and the presence of an activator in the preparations used in the quoted experiments cannot be eliminated. Human plasmin, which had been heated at acid reaction to remove any activators of plasminogen, showed no effect on human or bovine plasminogen, although a strong activity could be produced by a plasminogen activator in the presence as well as in the absence of plasmin; bovine plasmin, treated similarly, did not activate plasminogen present in a fibrin substrate (Müllertz 1955). These results indicate that the small increases sometimes observed in mixtures of plasmin and plasminogen preparations are due to the presence of activators of plasminogen in the samples, and that plasmin does not catalyse the conversion of plasminogen to plasmin.

The property of trypsin to activate plasminogen was observed independently and within a short period of time at four different laboratories. The activation of human plasminogen was demonstrated by means of a bovine fibrin substrate (Kocholaty, Ellis and Jensen 1952,

Lewis and Ferguson 1952 a) and a pig fibrin substrate (Jacobsson 1953). The activation of bovine plasminogen was demonstrated by means of a heated plasminogen-free fibrin substrate (Astrup and Sterndorff 1952 a). On the other hand only a low and erratic activation (Kocholaty et al 1952) and no activation (Lewis and Ferguson 1952 a) of bovine plasminogen by trypsin were reported. Further studies by Astrup and colleagues have shown that the activation of bovine plasminogen by trypsin is highly sensitive to even traces of antitrypsin, which are difficult to remove completely from bovine preparations (Astrup 1955). The activation of bovine plasminogen by trypsin has been confirmed by Jackson and Mertz (1954). Lewis and Ferguson (1952 a) presented a detailed investigation of the effect of trypsin on a purified dog blood plasminogen. The rate of activation was increased with increasing concentrations of trypsin, while the final amount of plasmin formed was proportionate to the concentration of plasminogen, but within certain limits independent of the trypsin concentration. Thus, trypsin probably produces plasmin by a proteolytic cleavage of the plasminogen molecule.

The effect of some other proteases on plasminogen has also been studied. Human and bovine plasminogen was not activated by chymotrypsin (Kocholaty et al 1952); and bovine plasminogen was not activated by chymotrypsin, an Aspergillus protease, a protease from B. subtilis (Astrup and Sterndorff 1952 a) or by chymotrypsin, ficin and papain (Jackson and Mertz 1954).

4. Activation of plasminogen by tissue

The early observations of the lytic effect of tissue cells on plasma clots have been reviewed by Goldhaber, Cornman and Ormsbee (1947), Permin (1949) and Lewis and Ferguson (1950). Astrup and Permin (1947) showed that this effect is caused by the transformation of plasminogen in the fibrin substrate into plasmin by an activator associated with the tissue fragments. Similar observations were reported independently by Goldhaber, Cornman and Ormsbee (1947).

The tissue activator is strongly attached to structural proteins. After crushing with sand and repeated washing and drying with alcohol and acetone, the dry powder retained most of the original activity (Permin 1947, 1949). The activator in suspensions of rat lung tissue (Tagnon and Petermann 1949), and dog lung tissue (Lewis and Ferguson 1950) was found to be present in all fractions obtained by differential

centrifugation, except in fragment-free supernatants. TAGNON and PETERMANN (1949) found it to reside especially in the microsome fraction. The activator could not be extracted from such preparations with saline, buffers, or dilute acids or bases (PERMIN 1947, 1949, LEWIS and FERGUSON 1950), but extraction was accomplished from the dry powder by means of potassium thiocyanate furnishing a soluble preparation (ASTRUP and STAGE 1952). Contrary to these observations HALSE (1948) and FANTL and FITZPATRICK (1950) reported that the fibrinolytic activity of human plasma was increased by saline extracts of brain tissue and ASTRUP and STERNDORFF (1952 a) succeeded in preparing a saline extract of ground tissue from human lung and pig brain, which contained a potent activator of bovine plasminogen. The tissue activator has been found in exceedingly variable amounts in different tissues from man, ox, pig, rabbit, rat, mouse, and calf embryo (PERMIN 1949, 1950) in almost all tissues from the dog and in lung fractions prepared from dog, mouse, rat, guinea pig, rabbit, cat and man (LEWIS and FERGUSON 1950). The activator produced plasmin in globulin solutions prepared by ammonium sulphate precipitation from human, bovine and dog serum (PERMIN 1949, 1950 b, ASTRUP, CROOKSTON and MACINTYRE 1950, LEWIS and FERGUSON 1950, TAGNON and PETERMANN 1949) and by precipitation at pH 5.3 and low ionic strength from human plasma (TAGNON and PALADE 1950). The enzyme attacked casein (PERMIN 1949, 1950 a, b) as well as fibrin. The activator was stable for several hours at 37°C at neutral reaction; it deteriorated markedly at 56°C at neutral reaction (PERMIN 1947, LEWIS and FERGUSON 1950, TAGNON and PALADE 1950) and at 45°C at acid or alkaline reactions (PERMIN 1949). PERMIN (1949) found that identical amounts of bovine plasmin were produced within the pH range 4.0-10.0, while an optimum of activation about neutrality was observed by TAGNON and Palade (1950). The mechanism of activation of bovine plasminogen by the tissue activator was studied by ASTRUP (1951 a). The total amount of enzyme obtainable after a complete reaction was shown to depend on the concentration of plasminogen as well as the activator. Small amounts of activator never produced as much plasmin as larger amounts, even if the reaction time was prolonged, and activator and plasminogen apparently combined in a reaction proceeding to an equilibrium. A complete conversion of plasminogen to plasmin was probably not obtained even with the most potent samples of the tissue activator. The activator was assumed to remove reversibly a blocking substance from plasminogen, thereby converting it into plasmin. The hypothesis implied that a sample of tissue activator should contain the blocking substance

after activation of plasminogen and that such samples should decrease the activity of plasmin solutions inversely proportionate to its remaining activator potency. This assumption was verified in several experiments with different samples (ASTRUP 1951 a). In other experiments samples of tissue activator inactivated plasmin to the same extent, even if they differed considerably in activator potency (ASTRUP 1951 b). Lewis and FERGUSON (1950, 1952 b) studied the activation of dog plasminogen by dog lung tissue activator. The rate and final yield of plasmin were dependent on the concentration of the activator and on the concentration of plasminogen. A complete activation was not obtained as compared with activation by staphylokinase. However, ASTRUP (1954 a) recently pointed out that the enzymic effect of trypsin on plasminogen suggests that the other activators of plasminogen act through an enzyme reaction and that discrepancies may be due to inhibitory agents.

5. Activation of plasminogen by bacterial filtrates

A substance is present in the culture filtrate from certain β-hemolytic streptococci, which does not attack casein, gelatin or peptone, but effects the lysis of clotted human plasma and fibrinogen (TILLETT and GARNER 1933, TILLETT, EDWARDS and GARNER 1934, GARNER and TILLETT 1934 a, b). It was originally assumed to be a fibrinolysin, specifically adapted for human fibrinogen and fibrin, but MILSTONE (1941) found that clots of highly purified human fibrinogen were not lysed by this factor and that a "lytic factor" in human globulin, in addition to the streptococcal factor, was necessary for the production of fibrinolysis. KAPLAN (1944) and CHRISTENSEN (1945) found that fibrinolysis and proteolysis were due to transformation of a protease precursor, plasminogen, into active enzyme, plasmin, by the streptococcal factor, streptokinase. Similar proteolytic activities were produced in different globulin preparations from human serum by streptokinase and by chloroform (KAPLAN 1944, RATNOFF 1948 a). The protease, which was formed by these two procedures, showed similar properties (CHRISTENSEN and MACLEOD 1945, RATNOFF 1948 a). The amount of proteolytic activity produced was found to be independent of the concentration of streptokinase, if the activation period was sufficiently long (CHRISTENSEN 1945). Under certain conditions the formation of plasmin, as estimated on gelatin, followed a first order reaction, the rate of which was proportionate to the concentration of streptokinase (CHRISTENSEN and MACLEOD 1945). Confirmative evidence was presented by REMMERT

and COHEN 1949, and it was concluded by these workers that the transformation of plasminogen to plasmin was catalysed by streptokinase. RATNOFF (1948 b) found that streptokinase combined stoichiometrically with plasminogen, when a crude globulin sample was used. Evidence of a catalytic reaction was obtained, when a heated plasminogen was used. The activity was estimated by means of casein. The difference was assumed to depend on the presence of inhibitors in the crude preparation. The purification and properties of streptokinase were studied by GARNER and TILLETT (1934 a), HOLMBERG and WINBLAD (1944), LOOMIS and SMITH (1946) and CHRISTENSEN (1945, 1947 b). Although human fibrinogen and fibrin were readily lysed by streptokinase, clots prepared with plasma or fibrinogen from animals were resistant. Thus, clots prepared from plasma of rabbit (TILLETT and GARNER 1933, MILSTONE 1941) rabbit and guinea pig (YEN 1935), ox (ASTRUP and PERMIN 1947), ox, rabbit, and horse (SCHMIDT 1936) and ox, pig, dog, rabbit, guinea pig, horse, sheep and some birds (MADISON 1934, DEVENTER and REICH 1934, LE MAR and GUNDERSON 1940, GERHEIM and FERGUSON 1949) showed no or only a slow lysis in the presence of streptococci or streptokinase. Similarly, no fibrinolytic activity was induced by streptokinase in globulin fractions prepared from ox serum (PERMIN 1947, ASTRUP and PERMIN 1948) and from dog and ox serum (LEWIS and FERGUSON 1950).

Certain staphylococci also produce a substance, staphylokinase, which effects the formation of a fibrinolytic and proteolytic enzyme in serum (LACK 1948, GERHEIM, FERGUSON, TRAVIS, JOHNSTON and BOYLES 1948). This substance differs in some respects from streptokinase. It lysed plasma clots from dog, man, guinea pig and rabbit, but failed to lyse those from ox, horse, sheep, rat and chicken. Furthermore, it required an incubation period of some length for complete activation as compared with the immediate effect of streptokinase on human material (GERHEIM and FERGUSON 1949); and the immediate, however, small effect on dog globulin (GERHEIM, LOGWOOD, ANDREWS, and WEITZENHOFFER 1951). LEWIS and FERGUSON (1951 a) presented a detailed study of the development of fibrinolytic activity by staphylokinase in globulin fractions as estimated by lysis time determinations on a bovine fibrin substrate. By far the highest fibrinolytic activity was obtained from the dog fraction, while human, cat, rabbit and guinea pig fractions showed moderate activities, and rat and ox fractions remained inactive. The rate of formation of fibrinolytic activity in a dog globulin fraction was proportionate to the concentration of staphylokinase, and the total activity obtained was dependent on the concentration of globulin and

independent of the concentration of staphylokinase. These results indicated that the reaction was catalyzed by staphylokinase.

The resistance of plasminogen in plasma and in globulin fractions from animals to streptokinase has received different explanations. Clots prepared from rabbit plasma were readily lysed by streptokinase on the further addition of human thrombin (TILLETT and GARNER 1933, MADISON 1934, JABLONOWITZ 1939). Addition of small amounts of human globulin made rabbit clots (MILSTONE 1941) and bovine clots (FERGUSON, TRAVIS and GERHEIM 1947) susceptible to streptokinase, indicating that the resistance was due to the absence of "lytic factor" in the clots. CHRISTENSEN (1945), however, obtained lysis of rabbit plasma clots with a concentrated, but not with a diluted solution of purified streptokinase, and presented evidence that the resistance of rabbit clots was in part due to the presence of a protease inhibitor. As this did not fully explain the resistance, he also suggested that rabbit plasminogen might be qualitatively different from human plasminogen or present in smaller concentrations. KAPLAN (1946 b) proposed that the resistance of clots to streptokinase might be due to (1) absence of plasminogen, (2) high levels of antistreptokinase, a specific immune antibody developed during streptococcal infections, or (3) high levels of an antiprotease, which was found to occur in especially high concentrations in animal sera.

However, none of these explanations seems satisfactory. The presence of a specific antistreptokinase should be insignificant in the presence of an excess of purified kinase and does not account for the effect of further addition of slight amounts of human thrombin or globulin. The activity against plasminogen-free protein substrates produced by streptokinase in human globulin was comparable to that occurring spontaneously or after staphylokinase activation in globulin fractions from dog, rabbit, guinea pig, monkey, and cat (CLIFFTON and DOWNIE 1950, CLIFFTON and CANNAMELA 1951, 1953 a, LEWIS and FERGUSON 1951 a) and to that occurring either spontaneously or after treatment with tissue activator or chloroform in globulin fractions from ox, and in many other animal sera. A high content of antiprotease may also be left out of consideration, as this inhibitor should be as active against plasmin produced by streptokinase as by other means. A strict species specificity of streptokinase also seems improbable, as small proteolytic and fibrinolytic activities have been produced by purified streptokinase in bovine fractions (ASTRUP, CROOKSTON and MACINTYRE 1950) in dog globulin (GERHEIM, LOGWOOD, ANDREWS and WEITZENHOFFER 1951) and in fractions from ox, pig, horse and chicken (CLIFFTON and CANNA-

MELA 1951, 1953 a). ASTRUP and colleagues especially studied human and bovine plasminogens by means of a bovine fibrin substrate. The fibrinolytic activity of bovine plasmin, activated spontaneously or by tissue activator, was compared with that of human streptokinase-activated plasmin. The results indicated that the concentration of plasminogen in human blood was 100-200 times higher than in bovine blood. Nevertheless, tissue activator produced insignificant activities in human plasminogen solutions diluted correspondingly (ASTRUP 1951 b); and the conclusion was drawn that two different plasminogens were present in human blood, one which could be activated by streptokinase and another by the tissue activator (ASTRUP and PERMIN 1947, ASTRUP 1948, ASTRUP, CROOKSTON and MACINTYRE 1950, ASTRUP 1951 b). CLIFFTON and CANNAMELA (1953 b) and JACOBSSON (1953) also observed a relatively much higher fibrinolytic than proteolytic activity of streptokinase-activated human globulin. Recent developments have contributed to the explanation of some of these discrepancies (see below).

Lysis of plasma clots was also induced by cultures of certain gas gangrene anaerobes (REED, ORR and SMITH 1941, REED, ORR and BROWN 1943). LEWIS, FERGUSON and JACKSON (1949) studied a large number of different bacteria, but only streptococci and staphylococci were found to produce kinase substances. The effect of gas gangrene anaerobes is probably due to proteases produced by these organisms.

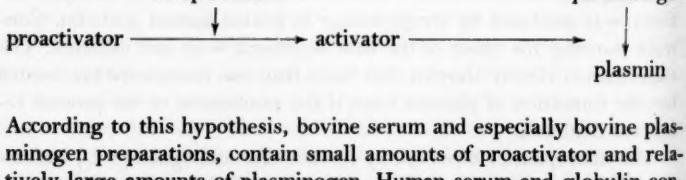
THE ACTIVATOR OF PLASMINOGEN IN BLOOD

1. Involvement of a proactivator-activator system in the formation of plasmin by streptokinase

Independent observations of GEIGER (1952 b), and MÜLLERTZ (1952 e) and MÜLLERTZ and LASSEN (1953) have shown that a factor besides plasminogen is needed for the formation of plasmin by streptokinase. GEIGER (1952 b) prepared mixtures containing (1) streptokinase, (2), the euglobulin fraction from human plasma, heated at 56°C for 1 hour, and (3) the euglobulin fraction from fresh serum of guinea pig, rabbit, chicken or duck. The proteolytic activity of the mixtures in all instances exceeded the sum of the amounts, which were produced on the addition of streptokinase to the separate globulin fractions. Similar results were obtained with heated human milk and saliva and bovine milk added to animal globulin. The heating was assumed to destroy a heat labile component, while a heat stable component remained, both being necessary for the formation of plasmin by streptokinase. The animal globulins were assumed to contain the heat labile but to lack the heat stable component. The heat labile component was assumed to be identical with or very similar to complement, since special treatment of human globulin or the addition of a number of substances resulted in a lower yield of plasmin by streptokinase, roughly parallel to the effect on complement. However, a considerable increase in proteolytic activity was produced by streptokinase in heated human globulin. Controls showing the effect of the heat treatment were not included. The experiments clearly showed that more than one component are needed for the formation of plasmin even if the mechanism of the process remained doubtfull.

The discrepancies observed by ASTRUP and colleagues (l.c.) indicating different proenzymes in human blood entailed a study of the properties and possible identity of bovine plasmin and of the agents produced by streptokinase in human blood and occurring in spontaneously lytic globulin obtained in vivo and post-mortem from human subjects. Most of the results of this investigation (MÜLLERTZ 1952 d, 1953 a, 1954 a)

were published in 1954. They indicated fundamental differences between the human and bovine active agents. MÜLLERTZ and LASSEN (1953) especially studied the effect of streptokinase on human and bovine globulin. They found that human streptokinase-activated globulin produced a strong lytic effect in a bovine plasminogen-containing fibrin substrate, but a relatively weak effect in a plasminogen-free fibrin substrate, prepared as described by LASSEN (1952). A very low proteolytic activity was produced by addition of streptokinase to human globulin or to bovine globulin, while a strong activity was obtained in a mixture containing streptokinase, minute amounts of human globulin, and bovine globulin. Bovine globulin was shown to contain large amounts of plasminogen, which could not be activated by streptokinase. A complete conversion was produced by the further addition of small amounts of human globulin. At a constant concentration of human streptokinase-activated globulin, the amount of protease formed was directly proportionate to the concentration of bovine globulin added. These experiments showed that streptokinase alone converted only a small part of the bovine plasminogen into plasmin, but a somewhat larger part in less purified than in purified bovine preparations. A factor, necessary for the formation of bovine plasmin by streptokinase, was probably present in insufficient amounts in bovine material and lost during purification. The activation of bovine plasminogen by human globulin and streptokinase demonstrated the formation of large amounts of an activator of plasminogen in human globulin by streptokinase. This was further supported by finding a similar activator in spontaneously active human blood (MÜLLERTZ 1953 b). The formation of plasmin by streptokinase was assumed to follow this scheme (MÜLLERTZ 1952 e, MÜLLERTZ and LASSEN 1953). The type of the reactions was not denoted:



According to this hypothesis, bovine serum and especially bovine plasminogen preparations, contain small amounts of proactivator and relatively large amounts of plasminogen. Human serum and globulin contain large amounts of proactivator and relatively small amounts of plasminogen. The experimental results were confirmed by SHERRY (1954). He found that streptokinase produced a weak proteolytic activity in bovine globulin and in dog and rabbit plasma; and that the addition of

small amounts of human globulin resulted in the development of a strong proteolytic activity. He did not think the hypothesis of a proactivator-activator system probable, but claimed that the activation of plasminogen was caused by a complex of streptokinase and human plasmin, or alternatively that it was due to a specific binding of a hypothetical inhibitor in animal sera by human plasmin, whereby animal plasminogen was released for activation by streptokinase. However, these complex theories do not supply an explanation of the activation of bovine plasminogen by active globulin from human blood obtained in vivo or post-mortem (MÜLLERTZ 1953 b). The non-involvement of human plasmin in the process was substantiated by MÜLLERTZ (1955). He prepared human plasmin free from streptokinase, activator and inhibitory substances and showed that human or bovine plasminogen was not activated by this preparation. Furthermore, a proactivator-activator system was demonstrated in human milk (ASTRUP and STERNDORFF 1953) and in human tears (STORM 1955) both of which materials contain no or little plasminogen. In a subsequent study, TROLL and SHERRY (1955) have adopted the hypothesis of a proactivator-activator system.

Further evidence has been supplied concerning the existence of a proactivator and a plasminogen in human blood. Attempts have been made to prepare human plasminogen free from proactivator. Although a complete separation has not been achieved, varying proportions between proactivator and plasminogen have been observed (MÜLLERTZ 1954 d). Trypsin, a bovine chloroform-activated plasmin, and different preparations of human streptokinase-activated globulin, plasma, and serum were assayed by means of casein, bovine fibrin and two synthetic esters of lysine and arginine (TROLL, SHERRY and WACHMAN 1954). A calculation of their data shows the relationship: fibrinolytic units/casein units to be 7 for trypsin, 18 for bovine plasmin and to vary from 1400 to 3800 for the different human globulin preparations and to be 8300 for human plasma and 10000 for human serum, activated by streptokinase. This clearly indicates different proportions between proactivator and plasminogen in the human preparations. The presence of two different esterase precursors in human globulin was suggested by TROLL, SHERRY and WACHMAN (1954), and established by TROLL and SHERRY (1955). In the latter study, one component was found to attack lysine ethyl ester (LEe) and another component to attack tosyl arginine methyl ester (TAMe). Studies on the inhibition of streptokinase activation of human globulin related the LEe esterase activity to the plasminogen activator and identified the TAMe esterase activity

with plasmin. LEe inhibited the activation of the enzyme responsible for both casein proteolysis and for TAMe esterase activity. The activation was inhibited by TAMe only at high concentrations. A competitive inhibition of the activator (related to LEe esterase) by LEe was found probable. The activation was also strongly inhibited by ornithine and lysine in accordance with previous observations by MÜLLERTZ (1954 a). Neither LEe or TAMe inhibited the formation of LEe esterase or of plasminogen activator. The activator was estimated by lysis time determinations on bovine fibrin. Addition of streptokinase to a spontaneously active human globulin increased the LEe esterase and the plasminogen-activating potency of the preparation, while no significant increase in TAMe esterase activity and proteolysis was produced.

CHRISTENSEN and SMITH (1950) found that the amount of streptokinase necessary for a complete activation of human globulin, as assayed by the lysis of bovine fibrin was 2 to 20 times higher than the amount, which was necessary, when the activity was assayed by casein. Accordingly, MÜLLERTZ (1955) obtained a complete activation of human plasminogen at concentrations of streptokinase about 50 times smaller than those used for a maximal formation of activator. In the same way activation of human globulin by varying concentrations of streptokinase revealed that the LEe and TAMe esterase activities bore different relations to the streptokinase concentration. (TROLL and SHERRY 1955). Heat denaturation studies by these workers on human globulin revealed different stabilities for the precursors of the LEe and TAMe esterase The TAMe esterase precursor showed a higher rate of deterioration at pH 2 and 100°C than the LEe esterase precursor. The opposite was observed at pH 9 and 50°C. A preparation was occasionally obtained which showed only LEe esterase activity after activation by streptokinase. Preparations showing only TAMe esterase activity were not obtained. This observation may indicate that plasmin attacks both esters. These studies clearly indicate that the LEe esterase activity is related to the activator. However, it is very peculiar that the relationship of LEe esterase activity to TAMe esterase activity and the relationship of fibrinolytic (i.e. activator) activity to proteolytic activity reported for different preparations of human globulin (TROLL, SHERRY and WACHMAN 1954) do not at all correspond. No explanation of this discrepancy has been proposed.

2. Involvement of a proactivator-activator system in the spontaneous formation of plasmin in blood

SCHMITZ (1936) originally suggested the presence in blood of a kinase for plasminogen. He obtained two fractions by elution of horse fibrin, both of which were proteolytic, and observed an increased proteolytic activity when the fractions were mixed. In all instances only a very slight proteolytic activity was obtained. LEWIS and FERGUSON (1951 b) prepared two fractions from normal dog blood, which remained inactive when stored separately, while a strong fibrinolytic activity developed after incubation of a mixture of the two fractions for several weeks at 5°C. Reproducing these results was reported to be difficult. MÜLLERTZ (1953 b) studied the strong fibrinolytic effects produced in a bovine fibrin substrate by globulin solutions prepared from spontaneously lytic blood obtained from human subjects after anoxaemic death. While strong fibrinolytic effects were produced in a normal plasminogen-containing bovine fibrin substrate, only weak effects were produced when the plasminogen of the substrate had been destroyed by heating. By addition of bovine plasminogen to a solution of active human globulin, 40 times more plasmin than originally present was formed. A similar, but lower increase in plasmin activity was obtained with active globulin obtained from human patients subjected to electrically induced convulsions in psychiatric therapy. The results were confirmed in experiments in which the lysis times were recorded. This was done with active globulin in bovine fibrin clots containing varying concentrations of bovine plasminogen. All these results indicate the presence of an activator of plasminogen in the active samples from human blood. In further studies (MÜLLERTZ 1954 c) the properties of different activator preparations were studied more closely. Three different activator preparations were compared, (1) a preparation made by addition of an excess of streptokinase to globulin from normal human blood, (2) a preparation made by addition of minimal amounts of streptokinase to globulin from normal human blood showing a similar plasminogen-activating potency as (3) a preparation of globulin from human fibrinolytic blood obtained post-mortem. The activator present in (1) was completely destroyed at acid reactions, while the activators present in the two other preparations showed a comparatively high stability at acid and characteristic and almost identical pH stability curves. The difference between the two streptokinase-activated preparations was assumed to depend on an incomplete conversion of proactivator into activator and a progressive formation of activator after the

heating procedure. The similar stability of the activator in (2) and (3) indicated the presence in spontaneously active blood of a factor with the same effect as streptokinase and a slow transformation of proactivator into activator in these samples also. The presence of this factor was demonstrated by the strong increase in activator activity produced by addition of normal globulin (i. e. proactivator) to different globulin preparations from active blood. In a large series of subsequent experiments, however, only slight increases in activator activity were produced by addition of proactivator, indicating that only small amounts of the streptokinase-like component and large amounts of proactivator were present (Müllertz 1954 d). Astrup (1954 b) also reported the presence in certain tissue extracts of small amounts of a factor which effected conversion of proactivator into activator. This substance is different from the tissue activator, which attacks plasminogen directly.

3. Methods of estimation of activator and proactivator

Astrup and Permin (1947) originally demonstrated that purified bovine fibrinogen contains large quantities of plasminogen. They showed that the lysis produced in a substrate of clotted bovine fibrinogen by tissue was caused by activation of the plasminogen of the substrate. These observations have been repeatedly confirmed by means of a tissue activator (Lewis and Ferguson 1950, Lassen 1952), an activator in urine (Astrup and Sterndorff 1952 b) and activators in human blood (Müllertz 1953 b, Müllertz and Lassen 1953). Purified bovine plasminogen was prepared by ammonium sulphate precipitation of the fluid obtained from purified fibrinogen after removal of fibrin by heat denaturation or clotting by thrombin (Astrup and Permin 1947, Müllertz 1953 b). A number of purified preparations of bovine fibrinogen (Ware, Guest and Seegers 1947, Jaques 1948, "Armour" bovine fraction I) were all found to contain large quantities of plasminogen (Astrup 1954 c). Sherry (1954) demonstrated the presence of plasminogen in "Armour" bovine fraction I, but did not refer to previous observations by other workers. He was able to prepare human and bovine fibrinogen free from plasminogen by repeated precipitations according to the technique described by Laki (1951). It can be concluded that lysis of clotted bovine fibrinogen is produced either directly by proteolytic (fibrinolytic) enzymes, which split the fibrin molecule, or indirectly by activators of plasminogen, which convert plasminogen in the substrate into plasmin with a subsequent splitting of fibrin. Both

plasmin and activators of plasminogen can be estimated by means of this substrate. PERMIN (1947, 1949, 1950 a) and ASTRUP (1948) originally designed the fibrin plate method for the estimation of the activator of plasminogen in tissue as well as the fibrinolytic enzyme of the blood. In this method, the active solutions are placed on the surface of a thin layer of clotted bovine fibrinogen, and the activity is measured by the area of the lysed zones. The method may be used as a convenient method for estimating other proteolytic enzymes (ASTRUP and ALKJÆRSIG 1952 a). The optimal conditions for the application of the method were studied by MÜLLERTZ (1952 a), and its accuracy and sensitivity were determined (MÜLLERTZ 1952 b, ASTRUP and MÜLLERTZ 1952). A detailed description of the method and its use was presented in the latter work. A quantitative estimation of the fibrinolytic activity of a diluted solution of streptokinase-activated human globulin was obtained by means of dilution curves and interpolation on a reference curve. It was found later (MÜLLERTZ and LASSEN 1953), that the lytic effect produced by this active material was caused almost exclusively by activation of plasminogen in the substrate and not as previously assumed by plasmin. However, this does not invalidate the results concerning the quantitative estimation and accuracy of the method. Further studies (MÜLLERTZ 1955) showed that the plasmin present in the highly diluted solutions of activator used in the method produced no significant lysis in the plasminogen-free bovine fibrin substrate prepared by heat denaturation of the fibrin as described by LASSEN (1952). Normal fibrin was found to be somewhat more susceptible than heated fibrin to activator-free human and bovine plasmin. The standard fibrin plate method was used for the assay of the activator of plasminogen in spontaneously lytic human blood (MÜLLERTZ 1953 b, 1954 a, b, c,) and in human streptokinase-activated globulin (MÜLLERTZ 1954 a, b, c, 1955). SHERRY (1954) confirmed the observation that the fibrinolytic assay for streptokinase-activated human plasmin, as generally employed with lysis time determinations on bovine fibrinogen clotted by thrombin, measures the activation of plasminogen in the substrate rather than the fibrinolytic activity of the human plasmin. He showed that the lysis of a plasminogen-free human and bovine fibrin produced by streptokinase-activated human globulin was comparable to that produced by bovine plasmin, trypsin, and chymotrypsin, when related to the respective effects on casein. The assay of lytic activity by lysis of fibrin clots was described in detail by CHRISTENSEN (1949) and applied in the determination of different components of the fibrinolytic system. It had been used extensively in many investigations before and has been widely used since

then. TROLL and SHERRY (1955) used the bovine fibrin – lysis time assay to determine the activator. However, this method is less satisfactory, since controls cannot be obtained showing the lytic effect of plasmin in different active samples. Such controls can be obtained in the fibrin plate method. TROLL and SHERRY (1955) found that the lysine ethyl ester esterase activity of streptokinase-activated human globulin was related to the activator, but no quantitative assay method was developed.

A theoretically correct estimation of the activator might be obtained (MÜLLERTZ 1955) by successive determinations of the amount of plasmin formed in a solution containing activator and an excess of bovine plasminogen. However, incompletely activated samples removed for the determination of plasmin by means of casein were found to be activated further and at an increased rate during the assay, allowing only approximate determinations of the plasmin concentrations in the activation mixture. Destroying the activator by adjusting the samples to pH 3 was attempted (MÜLLERTZ 1954 d) in order to stop the reaction and retain the plasmin. However, reproducible results were not obtained, probably because of adsorption of activator and plasmin to denatured protein formed by the treatment. An empirical method for the assay of the activator with appropriate controls was developed (MÜLLERTZ 1955). Only short reaction times were required. In this method activator was added to a solution containing bovine plasminogen and casein in excess and the mixture was incubated at 37°C for 20 minutes. By this procedure two reactions proceed simultaneously, the activation of plasminogen and the digestion of casein by the plasmin formed. The increase in optical density at 275 mμ of acid-deproteinized samples removed from the mixture was determined and converted into activator concentration by means of a reference curve. Proactivator was determined as activator after addition of an excess of streptokinase (MÜLLERTZ 1954 b).

4. Stability of the activator and proactivator

The stability of the activator at different reactions and temperatures was studied by means of the casein and fibrin plate methods (MÜLLERTZ 1954 c, 1955). The activator deteriorated almost completely, when kept at 22°C for 30 minutes at reactions below pH 5 or above pH 10. It showed an optimum of stability between pH 6.5 and 8.0. At neutral reaction it lost about 30% in 30 minutes at 37°, and about 1% at 22°. In the presence of its substrate, plasminogen, the activator also showed

a marked deterioration, when kept at pH 2.9 and 22°C for 30 minutes. The stability did not differ significantly in concentrated and in dilute solutions. The stability of the activator differed considerably from that of human plasmin (MÜLLERTZ 1954 c, 1955), and of human plasminogen (CHRISTENSEN and SMITH 1950, KLINE 1953), and of streptokinase (CHRISTENSEN 1947 b), especially at acid reactions. This difference is further evidence that the effect on bovine plasminogen of human streptokinase-activated globulin is caused by a substance different from previously known components of the proteolytic enzyme system of blood.

The observation that streptokinase and proactivator combine to form activator in a reversible reaction (see next section) should be considered in an evaluation of these experiments. The disappearance of plasminogen-activating potency from the preparation may be due to a displacement of the equilibrium by destruction of streptokinase or of the proactivator as well as by destruction of the activator itself. TROLL and SHERRY (1955) were able to destroy the plasminogen-activating potency of streptokinase-activated human globulin by a precipitation or a heat treatment at 100°C and pH 2 and to restore activator activity by addition of new streptokinase. They believed that streptokinase was destroyed by the treatment with a subsequent transformation of activator into proactivator. However, other observations contradict this assumption. Streptokinase was found to be very stable at pH 3 and 56°C for 60 minutes (CHRISTENSEN 1947 b), while the plasminogen-activating potency of streptokinase-activated globulin was lost almost completely at pH 3 and 22°C for 30 minutes (MÜLLERTZ 1954 c, 1955). After destruction of the plasminogen-activating potency at acid reaction (TROLL and SHERRY 1955) about 50% of the original amount of activator activity was restored by new streptokinase. In view of the high stability of proactivator under these conditions (MÜLLERTZ 1954 b, TROLL and SHERRY 1955), formation of almost the original amount of activator might be expected according to the concept of TROLL and SHERRY. No evidence was included in their study of a complete transformation of proactivator into activator by streptokinase, and the results may to some extent be explained by an incomplete transformation of proactivator, destruction of activator at acid reaction, and formation of more activator from proactivator hitherto not involved in the reaction. It is probable, however, that both activator, streptokinase and proactivator deteriorate at high temperatures at acid reactions. Further studies may reveal the relationship between the rates of deterioration and the actual stabilities of these different components.

The stability of the proactivator at different temperatures and reac-

tions was studied by MÜLLERTZ (1954 b). It showed a high stability at acid reactions losing only about 20% at 100°C and pH 3 for 30 minutes. A small loss in activity was observed at pH 7.1, 50°C for 30 minutes and it was completely destroyed at 70°C. Above pH 10 the proactivator was destroyed after 30 minutes at 50°C. Similar results were observed by TROLL and SHERRY (1955) in a study of the stability of the precursor of the LEe esterase (proactivator) at pH 2 and 100°, and at pH 9 and 50°.

5. Formation of the activator

Early studies by CHRISTENSEN and MACLEOD (1945) and REMMERT and COHEN (1949) had indicated a catalytic effect of streptokinase. WASSERMANN (1952) on the other hand found that streptokinase combined stoichiometrically with human plasminogen. He found the amount of plasmin formed from an excess of plasminogen to be dependent on the streptokinase concentration. At all levels of streptokinase maximal plasmin formation occurred almost instantaneously and did not increase with prolonged incubation. However, the activity was determined by lysis time determinations on clotted bovine fibrin and his results with this substrate are probably primarily pertinent to the formation of activator by streptokinase and proactivator. They support the assumption that these components react stoichiometrically. The destruction of the plasminogen-activating potency of streptokinase-activated human globulin at acid reaction, and the restoration of activator activity by additional streptokinase, led TROLL and SHERRY (1955) to suggest a reversible stoichiometric reaction between streptokinase and proactivator. Their observations strongly support the hypothesis of a reversible reaction, even if our present knowledge does not allow to account quantitatively for the results of the experiments

The reaction between streptokinase and proactivator was studied by MÜLLERTZ (1955) by means of a method for quantitative assay of the activator. At suboptimal concentrations of streptokinase only a partial transformation of proactivator into activator was obtained, followed by a deterioration of the activator formed according to a first order reaction. Significant amounts of antistreptokinase could not be demonstrated in the human globulin. The results contradicted a catalytic process, but indicated a stoichiometric reaction. In further experiments, the amount of activator formed with different proportions of streptokinase and proactivator was determined. By varying the concentration of streptokinase in the presence of a fixed concentration of proactivator, the

equivalent concentrations were found. In two experiments the concentration of streptokinase and of proactivator was varied above and below the equivalent concentration in the presence of a fixed concentration of the other component. The results of the two experiments were complementary and indicated that a reversible reaction proceeded to an equilibrium. The effect of dilution on the equilibrium was studied (1) by adding streptokinase to proactivator in equivalent concentrations and allowing the reaction to proceed to equilibrium in concentrated and dilute solutions, and (2) by diluting a concentrated solution containing the components in equilibrium. In the first case a slightly smaller amount of activator was formed in the dilute solution, while no difference was observed in the second experiment. A calculation of the theoretical effect of dilution on the equilibrium showed that the following reaction was the most probable:

$$\text{streptokinase} + \text{proactivator} \rightleftarrows \text{activator} + \text{inactive component}$$

Other observations (MÜLLERTZ 1954 b) may suggest an even more complicated reaction. The proactivator in normal human globulin and in active globulin prepared from post-mortem blood, showed a markedly different stability at different pH values and temperatures. Normal human globulin was found to yield an unchanged amount of activator on the addition of streptokinase after incubation for 20 hours at 22°C and pH 7.4, while active globulin yielded rapidly decreasing amounts of activator during incubation. The results indicated that a component, which was involved in the formation of activator by streptokinase, was consumed during the incubation. As the activator did not increase at the same time, this component was probably not identical with the proactivator. Furthermore, the amount of activator formed in a mixture of streptokinase, spontaneously active globulin and normal globulin, was considerably larger than the sum of the amounts of activator formed by streptokinase in separate samples of the globulins. This observation suggested that at least one of the samples contained proactivator, which was not converted completely into activator by addition of an excess of streptokinase. As a tentative explanation it was proposed that a reaction between streptokinase and another component in human globulin precedes the reaction with the proactivator, leading to the formation of activator. Other explanations may be proposed, however, and further studies are needed to decide this question.

Some observations, reported above, suggest that the mechanism of formation of activator from proactivator in spontaneously fibrinolytic human blood is similar to that described for streptokinase. Fractiona-

tion and purification of the different components present in the active and normal preparations should precede a study of the different reactions involved in this important process.

6. Formation of plasmin by the activator

Previous studies have led to different concepts of the mechanism of formation of human plasmin by streptokinase. CHRISTENSEN (1945), CHRISTENSEN and MACLEOD (1945) used gelatin, RATNOFF (1948 b) and REMMERT and COHEN (1949) casein, as substrates for the assay of plasmin; and these studies indicated convincingly that the conversion of plasminogen into plasmin was catalysed by streptokinase. WASSERMANN (1952) used a bovine fibrin substrate and found that streptokinase and plasminogen combined in a stoichiometric reaction, but his results with this substrate are pertinent primarily to the formation of activator by the interaction of streptokinase and proactivator. MÜLLERTZ and LASSEN (1953) demonstrated that at least two reactions were involved in the formation of plasmin by streptokinase. They concluded that, in view of the earlier observations, at least one of these must be a catalytic reaction. Their preliminary results indicated a stoichiometric reaction between activator and bovine plasminogen, but this conclusion is probably not valid because of the lability of the reacting components at the applied conditions (37°). TROLL and SHERRY (1955) confirmed the first order kinetics for the formation of human plasmin by streptokinase. They stopped the reaction by a precipitation technique before the assay of plasmin by tosyl arginine methyl ester and by casein. From this observation they suggested that activator catalysed the transformation of plasminogen into plasmin. As human material exclusively was used, the effect of activator on a proactivator-free plasminogen could not be investigated. Some information on the mode of action of activator on bovine plasminogen (free from proactivator) was presented by MÜLLERTZ (1955). The formation of bovine plasmin at different activator concentrations at 22° and 5° was followed with time. In all instances a progressive activation of plasminogen at small activator concentrations and a slow deterioration of plasmin resulted in approximately identical plasmin activities. The results suggested a catalytic reaction, but the deterioration of plasmin and the continuous formation of plasmin during the assay made it impossible to obtain conclusive evidence by these methods. Recent experiments by LASSEN (1956) have established the enzymic nature of the effect of activator on bovine plasminogen.

7. Effect of salts on the activation of plasminogen

The lytic effect in a bovine fibrin substrate produced by streptokinase-activated human globulin was considerably diminished at increasing concentrations of sodium chloride and ammonium sulphate (PERMIN 1949, MÜLLERTZ 1952 a). An increase in ionic strength of the fibrin substrate from 0.15 to 0.30 produced a 40% decrease of the lytic effect of this material, while the effect of trypsin was not affected (MÜLLERTZ 1952 a). Under the applied conditions, the observed effects may be related to the activation of plasminogen by the activator in the material, as well as to the subsequent splitting of the fibrin by plasmin, even if the results of the trypsin experiment may suggest that the activation is primarily influenced. The effect is probably not specific for the salts used. Thus, sodium, potassium, magnesium, barium and ammonium salts of hydrochloric, sulfuric, nitric and other inorganic acids influenced neither the lytic effect produced in a bovine fibrin substrate by an active dog globulin fraction, or the lytic activity produced in a dog globulin fraction by staphylokinase (LEWIS and FERGUSON 1951 a) and by trypsin (LEWIS and FERGUSON 1952 a). The effect of carboxylic and amino acids was studied by MÜLLERTZ (1952 d, 1954 a). Neutralized solutions of the different acids were added to solutions of bovine fibrinogen, and the fibrin substrates were prepared with these solutions. The ionic strength was kept constant at 0.15. Citrate increased markedly the lytic effect produced by human streptokinase-activated globulin, the increase being proportionate to the concentration of citrate within the range 0.002-0.018 M (MÜLLERTZ 1952 d). In the subsequent study (MÜLLERTZ 1954 a), this effect was studied more closely. The lysis produced by the activator of plasminogen in streptokinase-activated human globulin and in active globulin from postmortem human blood, was compared with that produced by bovine, spontaneously activated plasmin, trypsin, chymotrypsin, Aspergillus protease and a B.subtilis protease. A number of different carboxylic and amino acids were studied to find a possible chemical relationship between increased fibrinolysis and chemical structure. No effect was produced by addition to the substrate of a calcium precipitant (sodium fluoride), a divalent inorganic anion (sodium sulphate) and some organic compounds (glucose, lactose and glycerol). The effect of the activator in the human preparations was considerably increased by di- and tricarboxylic acids and decreased strongly by diaminomonocarboxylic acids, and the enhancing and inhibitory effects seemed to depend on the number of carboxylic and amino groups of each compound. The activity of the prote-

ases was generally not significantly influenced, but bovine plasmin was moderately inhibited by lysine and ornithine. These results suggest that the carboxylic acids influence only the activation process, while the amino acids possibly influence the activation process as well as the splitting of fibrin by bovine plasmin. Considerable variations were observed in experiments with trypsin, but a significant increase was produced by citrate. These observations may be explained by a varying susceptibility of the different batches of fibrin to the direct fibrinolytic and plasminogen-activating effect of this enzyme. GINSBURG, DE VRIES and KATCHALSKI (1952) found that lysis of human plasma clots (induced by addition of streptokinase, chloroform, or menstrual blood) was inhibited by polylysine, polyornithine and polyarginine. The monomers were ineffective under their experimental conditions. Polyaspartic acid (a monoaminodicarboxylic acid) obviated the antifibrinolytic effect of these basic amino acid polymers in vitro (GINSBURG, DE VRIES and KATCHALSKI 1952) and prevented their toxic manifestations in vivo (DE VRIES, FELDMAN, STEIN, STEIN and KATCHALSKI 1953). TROLL, SHERRY and WACHMAN (1954) demonstrated a competitive inhibition of the proteolytic effect of streptokinase-activated human globulin by addition of lysine ethyl ester (LEe) and tosylarginine methyl ester (TAMe) to the substrate. These observations indicated that the same enzyme centers were responsible for both actions. TROLL and SHERRY (1955) demonstrated a competitive inhibition of the activator of plasminogen (i. e. LEe esterase) by LEe, while TAMe probably did not inhibit activation of plasminogen. They confirmed the inhibition of the activator by lysine and ornithine.

8. Influence of fibrin and inhibitors on the activation of plasminogen in blood

Controversial results have been reported in studies on the splitting of fibrin and fibrinogen by different active preparations from blood. In some studies the decrease in acid-precipitable nitrogen (CHRISTENSEN 1945) and the increase in non-protein nitrogen (PERMIN 1949, 1950 b) have been determined. In the other studies the rates of splitting of fibrin and fibrinogen were compared by determining the length of time required for the lysis of fibrin and for rendering fibrinogen incoagulable by thrombin.

In a few experiments the effect of plasmin on purified preparations of fibrinogen and fibrin has been studied. PERMIN (1949, 1950 b) used a

bovine plasmin activated by tissue. RATNOFF (1953) used a bovine, chloroform-activated plasmin and a human plasmin prepared by successive treatments of globulin with streptokinase and chloroform. The bovine preparations and possibly also the human sample contain plasmin, but no or small amounts of activator. They produced almost the same rate of fibrinogenolysis as fibrinolysis in bovine and human purified preparations. It is thus highly probable that plasmin splits pure fibrinogen and fibrin at equal rates.

In most studies, streptokinase or preparations of activator have been added to plasminogen-containing solutions of fibrinogen or fibrin (i. e. clotted fibrinogen). Under these conditions the development and rate of fibrinogenolysis and fibrinolysis depend on the rate of activation of plasminogen as well as on the rate of the subsequent splitting of the substrate. In a number of these experiments fibrin and fibrinogen were lysed in almost the same length of time, indicating that the rate of activation of plasminogen as well as the rate of the splitting of the substrate were comparable in both instances. In other experiments, however, fibrin was split in a shorter length of time than fibrinogen. Thus, CHRISTENSEN (1945) reported identical rates of splitting of fibrin and fibrinogen on the addition of streptokinase to partially purified human preparations. Partially purified bovine fibrin and fibrinogen were found to be lysed in the same time on the addition of streptokinase-activated human globulin (MÜLLERTZ 1952 b), while RATNOFF (1953) found that a considerably higher rate of fibrinolysis than fibrinogenolysis was produced by a crude and a purified sample of human globulin, activated by streptokinase. In other studies spontaneously lytic human blood obtained post-mortem has been used. This material was found to induce fibrinolysis as well as fibrinogenolysis (MORAWITZ 1906, GOLOVANOVA 1950), and identical rates of splitting of purified bovine fibrinogen and fibrin were observed by MÜLLERTZ (1953 a). On the other hand, MOLE (1948) claimed it to be exclusively fibrinolytic and not fibrinogenolytic, although his observations on the effect of highly active samples are difficult to reconcile with this view. BIDWELL (1953) found that it produced a fast lysis of purified human fibrin but no splitting at all of human fibrinogen.

More uniform results have been observed when the splitting of fibrinogen and fibrin was studied in plasma, instead of in partially purified preparations. Thus, human streptokinase-activated globulin and plasma were found to induce fibrinolysis but no fibrinogenolysis in human plasma (FANTL and FITZPATRICK 1950, RATNOFF 1953); and the same results were observed with spontaneously lytic human globulin ob-

tained in vivo (RATNOFF 1953, BIDWELL 1953) and post-mortem (MÜLLERTZ 1953 a, BIDWELL 1953).

The fact that fibrinogenolysis was absent or very slow in the presence of plasma or serum, while under certain conditions it had been found to proceed at about the same rate as fibrinolysis in purified preparations led MÜLLERTZ (1953 a) to suggest that inhibitory substances might be responsible for the observed discrepancies. He made a qualitative estimation of the inhibitor content of different samples of post-mortem plasma, and found the rates of fibrinolysis and fibrinogenolysis to be almost identical in samples containing little or no inhibitor, while fibrinolysis, but no fibrinogenolysis, was observed in samples with a high inhibitor content. It may be pointed out now, that the different rates of fibrinogenolysis and fibrinolysis may be explained by an influence of inhibitors on the activation of plasminogen as well as on the action of plasmin. However, a number of studies have indicated that the influence of fibrin and of an inhibitor on the activation process is the primary cause of the observed discrepancies.

Fibrin seems to be of special importance for the activation of plasminogen. The early studies of NOLF (1908) showed the significance of the presence of fibrin for the development of fibrinolysis. HOUGARDY (1933, 1934) studied the spontaneous and chloroform-induced lysis of fibrin and plasma clots, and demonstrated the adsorption of the lytic agents to fibrin. The activator present in post-mortem active globulin was found to be strongly adsorbed to fibrin and released again after lysis of the clot (MÜLLERTZ 1953 a). The activator in active globulin from living human subjects (MÜLLERTZ 1953 b) and in streptokinase-activated human globulin (MÜLLERTZ 1954 d) was also found to be strongly adsorbed to fibrin. The "plasma active fibrinolysin" shown to be adsorbed to fibrin by FEARNLEY (1953) is probably identical with the activator. RATNOFF (1953) concluded that plasmin digested fibrin and fibrinogen at equal rates, while fibrin was destroyed much faster than fibrinogen in spontaneously lytic human blood and on the addition of streptokinase to human plasma. He suggested that the activation of plasminogen was accelerated by the process of coagulation, possibly through an effect of the fibrin surface.

The presence of an inhibitor against the activation of plasminogen has been suggested by SCHMITZ (1937), HULTIN and LUNDBLAD (1942) and LEWIS and FERGUSON (1951 b). The presence of an antiactivator in blood was demonstrated in preliminary experiments by MÜLLERTZ (1954 c). He found that a crude sample of bovine globulin inhibited the activation of bovine plasminogen by the activator in human streptoki-

nase-activated globulin, and further experiments showed that the inhibitory effect of bovine globulin on the activation process was markedly reduced in the presence of fibrin. BIDWELL (1953) followed the progressive lysis of fibrin clots in diluted samples of active human plasma obtained in vivo. She found that the amount of fibrin remained constant for some hours, and then began to diminish rapidly, the lysis being complete in a relatively short time. All these observations indicate that plasmin is not originally present in spontaneously lytic blood and that plasmin is not formed by addition of activator to plasma or to preparations with a high inhibitor content. In fluid blood the activator is probably bound by antiactivator and consequently no plasmin is formed and no splitting of fibrinogen takes place. In the presence of fibrin, activator is adsorbed to the fibrin surface, whereby the effect of the antiactivator is reduced; and the activation of plasminogen with subsequent fibrinolysis occurs.

It is possible that the action of plasmin on fibrinogen and fibrin is influenced by fibrin and inhibitors in a similar way as the activation of plasminogen. Bovine spontaneously activated plasmin was found to be adsorbed to fibrin as opposed to trypsin, chymotrypsin and some other proteases (MÜLLERTZ 1954 d). It has been pointed out above, that plasmin probably splits pure fibrinogen and fibrin at equal rates. The chloroform-activated human plasmin used by BIDWELL (1953) hardly contained any activator; nevertheless, it destroyed human fibrin approximately 2.5 times as fast as human fibrinogen. Some antiplasmin may have been present in the human fibrinogen used in this experiment. Plasmin is probably bound by antiplasmin in blood so that no proteolytic effect is disclosed. In the presence of fibrin, the effect of antiplasmin may be reduced by the adsorption of plasmin to fibrin so that some lysis of fibrin may occur. At present experimental evidence is lacking concerning the effect of preparations of activator-free plasmin on fibrinogen and fibrin in the presence of antiplasmin in plasma, and further studies are needed to establish the nature of the relationship between plasmin, antiplasmin and fibrin.

9. The proactivator-activator system and previous observations

Activation of plasminogen in the organism. It is probable that most previous observations on low and moderate fibrinolytic activities of plasma or globulin fractions obtained from living and dead human sub-

jects have been concerned not with plasmin but with the activator of plasminogen, or perhaps also with a system of components interacting to form activator. This concept supplies an explanation of the strong lytic activity produced by spontaneously active samples diluted by means of human fibrinogen (with plasminogen) (MACFARLANE and PILLING 1946). FANTL and SIMON (1948) found no inhibition of the fibrinolytic activity, by addition of normal plasma to active plasma. BIDWELL and MACFARLANE (1951) and BIDWELL (1953) found the fibrinolytic activity of such mixtures to depend only on the proportion of lytic plasma, no inhibition being apparent; although a strong inhibition of chloroform-activated human plasmin was produced by normal and lytic plasma. The results may simply mean that varying rates of activation of plasminogen are produced in the clot by varying amounts of activator available in the mixtures, the effect of the inhibitors being insignificant in the presence of the clot. Fibrinolytic plasma clots made in the presence of calcium lysed less rapidly than those made in its absence (FEARNLEY and TWEED 1953, BIDWELL 1953). The curves showing the progressive lysis of such clots (BIDWELL 1953) suggest that the activation of plasminogen in the presence of clots made with calcium is retarded, while the subsequent lysis of the clots proceeds at the same rate in the presence and in the absence of calcium.

Activation of plasminogen by fractionation, chloroform and proteases. It may reasonably be assumed that the activator of plasminogen is involved in the spontaneous activation of plasminogen after fractionation and after chloroform treatment. As already proposed by SCHMITZ (1937) and by LEWIS and FERGUSON (1951 b) these procedures may remove or destroy inhibitors both of the free enzyme or of a kinase of plasminogen. The formation of plasmin after chloroform treatment of plasminogen led CHRISTENSEN (1947 a), ROCHA E SILVA and RIMINGTON (1948) and RATNOFF (1948 b) to suggest an autocatalytic reaction. Their observations could indicate a simultaneous formation of activator and a catalytic effect of the activator on plasminogen. Some spontaneously activated human preparations contain activator (MÜLLERTZ 1954 d) as well as plasmin, but most observations indicate that no excess of activator is present in preparations activated spontaneously or after chloroform treatment. Thus, a highly purified fraction prepared by ethanol precipitation from human serum showed proteolytic activity, but no excessive lytic activity on a bovine plasminogen-containing substrate (SHERRY 1954), and thus contained no surplus of activator.

Activation of plasminogen by tissue. At present nothing indicates an involvement of the proactivator-activator system in the activation of

plasminogen by tissue. The activator in tissue apparently attacks plasminogen directly. It may be noted that the tissue activator was found to react stoichiometrically with plasminogen as opposed to the catalytic transformation of plasminogen produced by trypsin and by activators in blood. However, ASTRUP (1954 a) recently pointed out that, since the effect of trypsin on plasminogen must be assumed to be a catalytic reaction, it is possible that the other plasminogen activators also act through an enzymic process, and that discrepancies in a number of experiments are caused by the presence of inhibitory agents.

Activation by bacterial filtrates. The resistance of clots and plasminogens prepared from different animal sera may be explained by a low content of proactivator and perhaps also high concentrations of inhibitors, especially antiactivator, in animal blood. It has been pointed out, that the relatively high fibrinolytic activity, produced by human streptokinase-activated material in a bovine fibrin substrate, is caused by activation of the plasminogen in the substrate. Consequently, previous studies on the interaction of streptokinase, human plasminogen and plasmin, performed by means of this substrate, are primarily pertinent to the proactivator-activator system and only to a very slight degree to plasminogen and plasmin. This especially applies to the fibrinolytic methods generally used for the assay of the activity of these factors (CHRISTENSEN 1945, ASTRUP and MÜLLERTZ 1952, FLECHER 1954). The belief that the fibrinolytic assay estimated plasminogen, while actually proactivator was determined, may have led to wrong conclusions. Thus LEPOW, WURZ and PILLEMER (1954) describe the requirement of a factor for the inactivation of complement by plasmin. Streptokinase was added to human serum, which had been treated in different ways, and the fibrinolytic activity was compared with the effect on complement in the serum. As complement is destroyed by plasmin (PILLEMER, RATNOFF, BLUM and LEPOW 1953) the required factor may simply be plasminogen, which had been destroyed by treating the serum in different ways.

Further studies are needed to determine the mechanism of action of staphylokinase. The proteolytic and fibrinolytic activities produced in globulin from dog, rabbit, guinea pig (CLIFFTON and CANNAMELA 1951, 1953 a) suggest that staphylokinase is a direct activator of plasminogen, while the results obtained with globulin from man, chicken, cow, horse, pig, and rat suggest that the mechanism is similar to that in streptokinase experiments.

PLASMINOGEN AND PLASMIN

1. Preparation of plasminogen and plasmin

There is a considerable accumulation of evidence that the various proteolytic, fibrinolytic and fibrinogenolytic effects produced by different preparations from blood are ultimately caused by a single enzyme, plasmin. Almost without exceptions, previous discrepancies indicating the probable presence of different proenzymes and enzymes in blood may now be explained by the formation and presence of an activator of plasminogen in the active samples and by the presence of plasminogen in the substrates used for the assay of the activity. Correct information about the properties of plasminogen and plasmin can be obtained only from studies in which the activity of the preparations has been estimated by plasminogen-free substrates. Gelatin, casein (CHRISTENSEN 1945, KAPLAN 1944 and many others) and hemoglobin (KAPLAN 1944, GROB 1949) may be mentioned among the different proteins used for the assay of plasmin. The proteolysis has been determined by the decrease in viscosity of gelatin, the decrease in acid-precipitable nitrogen, and liberation of amino nitrogen (CHRISTENSEN 1945). The increase in tyrosine- and tryptophane-containing low molecular compounds has been estimated by means of a phenol reagent (CHRISTENSEN 1945, REMMERT and COHEN 1949) and by the increase in optical density at 275 mμ of the acid-deproteinized solutions (MÜLLERTZ 1955). The assay of plasmin by a heated, plasminogen-free, bovine fibrin substrate has been described by LASSEN (1952). Synthetic esters of basic amino acids have also proved to be suitable substrates for plasmin (TROLL, SHERRY and WACHMAN 1954, SHERRY 1954, TROLL and SHERRY 1955). The way in which the preparations have been made is of significance in evaluating the properties of plasmin. It is important to know whether in addition to plasmin, they contain plasminogen, activating substances and inhibitors. An increase in activity of such preparations may depend on a destruction of inhibitory substances with the liberation of additional free enzyme or a further activation of unconverted plasminogen. The influence of different conditions and substances on plasmin in such pre-

parations cannot be safely evaluated. The many different methods for activating plasminogen have been reviewed above. Some of these procedures are preferable in the preparation of plasmin. A crude plasminogen used for the preparation of human plasmin has been obtained by precipitating the euglobulin fraction from human serum at pH 5.3 and low ionic strength. This procedure was originally described by MILSTONE (1941) and has been extensively used since then. Crude fractions have also been prepared by precipitation of the globulin fraction by ammonium sulphate and by a combination of these and similar procedures (MILSTONE 1941, CHRISTENSEN 1945, ROCHA E SILVA and RIMINGTON 1948, PERMIN 1949 and others). A purified preparation was obtained by a low salt, low temperature, ethanol separation technique (ONCLEY et al 1949) (fraction III-3). This fraction contains active plasmin as well as the split products of fibrin. A purified human plasminogen was also obtained by successive isoelectric precipitations followed by adsorption to and elution from kaolin (REMMERT and COHEN 1949). CHRISTENSEN and SMITH (1950) obtained a further purification by successive extractions of human fraction III (COHN et al 1946) in 0.2 n sulfuric acid followed by precipitation at pH 5.2 and low ionic strength. KLINE (1953) modified and extended this method of preparation and obtained a concentration of enzyme more than 400 times higher than that of serum. From solutions of this purity, a crystalline fraction containing plasminogen was readily obtained. However, streptokinase-activated preparations of CHRISTENSEN and SMITH (1950), REMMERT and COHEN (1949), and KLINE (1953) all showed a relatively much higher activity against bovine plasminogen-containing fibrin than against casein (TROLL, SHERRY and WACHMAN 1954), a fact which indicates the presence of considerable quantities of proactivator in the preparations. The absence of inhibitors from these purified preparations has not been established. The intense co-precipitating property of plasminogen has been the principal obstacle to its purification and is the probable reason for the difficulty in separating plasminogen from proactivator. Human plasmin has generally been prepared from the plasminogen preparations by activation with streptokinase. The large quantities of proactivator in all human preparations entail a complete activation of plasminogen by addition of small amounts of streptokinase (MÜLLERTZ 1955). The active preparations contain streptokinase, activator and in some instances also inhibitors in addition to plasmin. Activation of human plasminogen by chloroform has not yielded a complete activation, as further increases in activity could be produced by addition of streptokinase (RATNOFF 1948 a, GROB 1949, CHRISTENSEN

1947 a). A plasmin preparation (MÜLLERTZ 1955) was made by streptokinase activation of a crude globulin fraction, and the active material was purified by heating at 100°C and pH 1.8 for 30 minutes. This treatment was found to destroy streptokinase, activator and almost all inhibitor, with a moderate loss of plasmin. A further purification was obtained by precipitation at pH 5.3 and low ionic strength. The preparation contained relatively much protein but was completely activated and free from activating substances and inhibitors. It would be more satisfactory, however, to apply this procedure to a more purified plasminogen.

Crude plasminogen preparations have been obtained from animal sera by isoelectric precipitation, ammonium sulphate precipitation, and by a combination of both procedures (TAGNON, DAVIDSON and TAYLOR 1942, PERMIN 1947, 1949, LOOMIS, GEORGE and RYDER 1947 and others). Purified bovine preparations have been made from purified fibrinogen from which the fibrin was removed after clotting by thrombin or by a heat denaturation (ASTRUP and PERMIN 1947, MÜLLERTZ 1953 b). Bovine plasmin has generally been prepared by chloroform activation; and in a considerable number of studies, samples prepared as described by Loomis, GEORGE and RYDER (1947) have been used. However, it is very unlikely that chloroform produces a complete activation of bovine plasminogen. Thus, PERMIN (1949) obtained only appreciable activities in 10-20% of the samples treated with chloroform, although considerable activities consistently were obtained by activation with a tissue activator. More plasmin was produced in a bovine plasminogen by tissue activator than by chloroform (ASTRUP 1951 b). Furthermore, a complete activation of bovine plasminogen was probably not obtained even with the most potent samples of tissue activator (ASTRUP 1951 a). Activation by trypsin has produced highly variable results. The low content of proactivator in bovine plasminogen preparations is the cause of the very weak activity produced by streptokinase. A complete activation of a purified bovine plasminogen was obtained, however, by addition of an excess of the activator of plasminogen formed by streptokinase in human blood (MÜLLERTZ 1955). The preparation was freed from activator by incubating at 22° at pH 3 for 30 minutes with only a small loss of plasmin. The content of human plasmin of the samples was about 3%. By this procedure it is possible to prepare plasmin from different animal species and to investigate and compare their properties.

2. Properties of plasmin

Plasmin is a proteolytic enzyme, which splits casein, gelatin, hemoglobin, fibrinogen and fibrin. No specificity of plasmin against fibrin is probable, as the relation between the effect of plasmin against a plasminogen-free fibrin and casein was found to be almost identical with that of trypsin (SHERRY 1954). Plasmin has been observed to destroy the clotting components of blood, accellerin (LEWIS, HOWE and FERGUSON 1949) and prothrombin (SEEGERS and LOOMIS 1946, LOOMIS, GEORGE and RYDER 1947), while thrombin was not found to be attacked (LOOMIS, GEORGE and RYDER 1947, SEEGERS and LOOMIS 1946, GLAZKO 1947). Plasmin also destroys complement (PILLEMER, RATNOFF, BLUM and LEPOW 1953) and vasopressin (CROXATTO, BADIA and CROXATTO 1948). Plasmin and trypsin are distinctly different enzymes. KAPLAN (1946 a) found that streptokinase did not activate trypsinogen, nor did enterokinase activate plasminogen. Plasmin was found to produce primary splitting products of a high molecular weight from fibrinogen, fibrin and casein (HOLMBERG 1944, SEEGERS, NIEFT and VANDENBELT 1945, KAPLAN 1954). The further splitting of these compounds was probably slow (KAPLAN 1954). CHRISTENSEN and MAC LEOD (1945) showed that trypsin was able to continue the hydrolysis of casein after hydrolysis by plasmin was practically complete, while no further hydrolysis was produced on the addition of plasmin to casein, which had been previously digested by trypsin. A more extensive splitting of fibrinogen, fibrin and casein by trypsin than by plasmin was reported by PERMIN (1949, 1950 b).

The pH of optimum activity of plasmin has been established against different substrates. Human and bovine plasmin showed nearly identical pH activity curves against casein with an optimum in the pH range 7.5 – 8.3 (MÜLLERTZ 1955). Similar results have been reported previously with a plasmin prepared by elution from horse fibrin as estimated on horse fibrin (SCHMITZ 1936), with a bovine plasmin activated by chloroform as estimated on gelatin (KAPLAN, TAGNON, DAVIDSON and TAYLOR 1942), and with human plasmin activated by streptokinase or chloroform as estimated on casein (RATNOFF 1948 a, REMMERT and COHEN 1949), or gelatin (CHRISTENSEN and MACLEOD 1945).

Reports on the stability of plasmin have been contradictory. CHRISTENSEN and MACLEOD (1945) found a maximum of stability of human plasmin at pH 7.0 – 7.4 with a rapid loss above and below these values in the pH range 3.5 – 11. On the other hand a remarkably high stability of human plasmin at acid reactions was established by MÜLLERTZ

(1954 a, 1955). Only about 60% of the activity was lost after heating at 100°C and pH 1.8 for 30 minutes. The high stability at these conditions was established in experiments using both impure and partially purified preparations and both with casein and heated fibrin as substrates. SHERRY (1954) also noted a high stability of human plasmin at pH 2. CHRISTENSEN and MACLEOD (1945) used rather crude preparations of streptokinase and human globulin, and the activity was assayed by means of viscosimetry on gelatin. An incomplete activation or loss of plasmin by adsorption to protein denatured by heating at acid reaction and removed before the assay may be considered as an explanation of their observations. At neutral reaction, CHRISTENSEN (1945) found human plasmin activated by streptokinase to be fairly stable, while RATNOFF (1948 b) found it to be rather labile, though the stability was greatly increased after heat or chloroform treatment. KLINE (1954) found that plasmin prepared by streptokinase activation of a purified human globulin fraction was labile at neutral pH and 35°C, but was stabilized considerably in the presence of casein and synthetic esters (TROLL, SHERRY and WACHMAN 1954).

The stability of bovine plasmin was studied by MÜLLERTZ (1955). It showed a high stability at acid reactions especially at pH 3. About 30% remained after heating at this reaction and 100°C for 30 minutes. At neutral reaction bovine plasmin was rather labile, losing about 50% of its activity in 30 minutes at 37°. In the presence of casein it was completely stable for 20 minutes at 37°C. The stability curve at the pH range 5.0 – 9.5 was approximately the inverse of the pH activity curve. LEWIS and FERGUSON (1951 a) found a similar stability of a spontaneously activated dog blood plasmin in the pH range 5.0 – 9.7 and 37°C, as estimated on a bovine fibrin substrate. KAPLAN, TAGNON, DAVIDSON and TAYLOR (1942) found bovine plasmin activated by chloroform to be rather labile at neutral reaction. The results indicate that both human and bovine plasmin are autodigestive. Thus the rate of autodigestion should be high in purified preparations, while a competitive inhibition of this effect should occur in the presence of added protein or after heat or chloroform treatment, which makes contaminating serum globulins more susceptible to plasmin.

MECHANISM OF FIBRINOLYSIS IN BLOOD

The many different observations published recently suggest a change in concept concerning the mechanism of fibrinolysis under physiological conditions in blood. Observations on the properties and mechanism of interaction of some components have shown that terms now generally used are not suitable. The stoichiometric reaction of streptokinase with a component in blood makes the suffix "kinase" unsuitable. A factor similar in effect to streptokinase was found in tissue and in active blood, and may possibly be released simultaneously with tromboplastic substances. The term *lysoplastin* is therefore proposed for substances, which react with one or more components in blood to form an activator of plasminogen. Streptokinase accordingly may be termed *streptococcal lysoplastin* and the components in tissue and in active blood *tissue lysoplastin* and *blood lysoplastin*. The activator of plasminogen is probably a proteolytic enzyme specifically adapted for plasminogen and the term *plasminogenase* seems appropriate. The inhibitor of this enzyme in blood may be called *antiplasminogenase*.

Some studies suggest that the development of fibrinolytic activity in blood is associated with the state of the vascular system. It is probable that any change, which leads to an increased permeability of the vascular endothelium and release of tissue components, at the same time entails an increase of the potential clotting tendency and of the potential fibrinolytic activity of blood. A factor from tissue apparently combines with components in blood to form plasminogenase. Blood from normal human subjects has been found to contain a potential fibrinolytic activity, which is increased under certain conditions. It is probable that some plasminogenase is continously formed by small amounts of lysoplastin released into blood, so that small amounts of plasminogenase is present in blood under normal conditions. In fluid blood, all active substances circulate in a neutralized state. Consequently, there is no activation of plasminogen and no splitting of fibrinogen or of other plasma proteins. Plasminogenase is bound by a specific inhibitor, anti-

plasminogenase. It is strongly adsorbed onto fibrin from the fluid circulating blood. In this way plasminogenase accumulates on the surface of fibrin deposits, and local high concentrations can be reached, although the concentration of plasminogenase in the blood is very low. The effect of inhibitors against activation and perhaps also against the effect of plasmin is reduced by the adsorption of activator and plasmin to the fibrin substrate. The plasminogenase-antiplasminogenase compound may dissociate on the adsorption of plasminogenase onto fibrin. The activation of plasminogen takes place on the fibrin surface, and the fibrin is subsequently digested by the plasmin formed in the clot. By the lysis of the fibrin, plasmin and plasminogenase are released into blood and probably neutralized there by their respective inhibitors. Simultaneously some deterioration of the rather labile components takes place.

The various stoichiometric and catalytic reactions involved in the activation of plasminogen and in the effect of plasmin in blood, are presented in the following schemes. They may serve as a working hypothesis for further investigations:

I. *Equilibria in fluid blood.*

(1) lysoplastin + proplasminogenase $\rightleftarrows$ plasminogenase + inactive component.

(2) plasminogenase + antiplasminogenase $(\rightleftarrows)$ bound plasminogenase.

(3) plasmin + antiplasmin $(\rightleftarrows)$ bound plasmin.

Lysoplastin may react with another component before the formation of plasminogenase from proplasminogenase takes place.

II. *Equilibria and enzymic reactions in the presence of fibrin.*

Plasminogenase is adsorbed from circulating blood onto fibrin:

(4) plasminogenase + fibrin $(\rightleftarrows)$ fibrin-plasminogenase

and hence:

(5) bound plasminogenase $(\rightleftarrows)$ plasminogenase + antiplasminogenase.

On the fibrin surface:

(6) plasminogen $\xrightarrow{\text{plasminogenase}}$ plasmin

and:

(7) fibrin $\xrightarrow{\text{plasmin}}$ protein fragments.

It is a further possibility that fibrin also influences plasmin and antiplasmin in a similar way:

(8) plasmin + fibrin $(\rightleftarrows)$ fibrin-plasmin
and hence:
(9) bound plasmin $(\rightleftarrows)$ plasmin + antiplasmin.

It is probable that fibrin deposits are continuously formed in various places in the organism also under physiological conditions and that these deposits are continuously and rapidly lysed. In certain pathological states, especially chronic infections, cancer and other wasting diseases the clotting tendency of blood is increased. Simultaneously the inhibitors of the fibrinolytic components are increased, whereby physiological fibrinolysis is reduced. This may lead to thrombosis. In other states, excessive fibrinolysis occurs. This is often associated with a simultaneous decrease in inhibitor content of blood and may lead to the destruction of fibrinogen and other clotting components with a resulting incoagulability of blood, a haemorrhagic diathesis and a serious often fatal outcome.

The present knowledge of the proteolytic enzyme system in blood is fragmentary. It is important to note, that the various reactions occurring under normal conditions in blood in the organism can be demonstrated only by application of specific procedures, e. g. the production of a fibrin clot, dilution or fractionation. It is difficult, therefore, to obtain a precise knowledge of the state of the different components of the system in fluid blood in the vessels. Further studies are needed to establish the properties and the mechanism of interaction of the components already known to exist. Future investigations may possibly reveal other components and other mechanisms involved in the sequence of events, which ultimately produce the lysis of the fibrin clot.

SUMMARY

Activation of plasminogen.

Plasmin is the proteolytic and fibrinolytic enzyme of blood. It is normally present in blood as an inactive precursor, plasminogen. In the living human organism an increased fibrinolytic activity develops under conditions of emotional and physical stress. In severe cases, afibrinogenaemia, incoagulable blood and haemorrhagic diathesis occur. After death, strong fibrinolytic activities are often found in blood. An increased vascular permeability and release of tissue components into the blood seem to be important factors in the development of fibrinolysis in the organism. In vitro, a conversion of plasminogen into plasmin can be effected by precipitation procedures or by treating serum or globulin fractions with chloroform, tissue or trypsin. Culture filtrates from certain streptococci and staphylococci contain substances, streptokinase and staphylokinase, which effect the activation of plasminogen. By precipitation of the globulin fraction or by a chloroform treatment inhibitors in serum are removed or destroyed. Tissue activator has been found to react stoichiometrically with plasminogen in a reversible reaction. It is now certain that plasmin does not activate plasminogen, however, trypsin possesses this property. Streptokinase effects the conversion of human plasminogen into plasmin, but animal plasminogens are activated only to a slight degree, or not at all. This and a number of other observations have indicated that the activation of plasminogen is more complicated than previously assumed.

The activator of plasminogen in blood.

Recent investigations on the effect of streptokinase on human and bovine plasminogen have shown that streptokinase does not react directly with plasminogen, but indirectly through a proactivator-activator system. Further studies on the mode of reaction have shown that streptokinase reacts with proactivator in a reversible reaction:

$$\text{Streptokinase} + \text{proactivator} \rightleftarrows \text{activator} + \text{inactive component.}$$

Streptokinase may react with another component in blood before the formation of activator from proactivator takes place. Studies on the effect of activator on bovine proactivator-free plasminogen have shown that the activator is a proteolytic enzyme which catalyses the conversion of plasminogen to plasmin:

$$\text{Plasminogen} \xrightarrow{\text{activator (plasminogenase)}} \text{plasmin}$$

A proactivator-activator system is probably involved in the spontaneous formation of plasmin in vivo and vitro. A potent activator of plasminogen has been found in spontaneously lytic human blood and a factor with an effect similar to streptokinase has been demonstrated in such samples and in certain tissue extracts.

The presence of a proactivator in addition to plasminogen in human blood is supported by studies on the stability of the precursors of the lysine ethyl ester esterase and tosyl arginine methyl ester esterase in human blood.

These observations supply an explanation of a number of previous discrepancies. The refractoriness of bovine preparations to streptokinase is due to a low content of proactivator. Human preparations contain a large amount of proactivator, and streptokinase-activated and spontaneously lytic human preparations from blood contain considerable quantities of an activator of plasminogen in addition to plasmin. The pronounced lytic effect of these samples on the generally used bovine fibrin substrate is due almost exclusively to activation of the plasminogen, which contaminates this substrate. Most previous observations on the fibrinolytic activity of blood from living and dead human subjects and of streptokinase-activated human preparations from blood have been concerned with the activator and not with plasmin.

The existence of a proactivator-activator system is also of significance for the concept of the development of fibrinolysis in blood. In purified preparations, the splitting of fibrinogen and fibrin, produced by plasmin and activator, occurs at almost identical rates. In plasma, in the presence of inhibitors, only fibrin, but not fibrinogen, is split. The activator is strongly adsorbed to fibrin. An antiactivator is present in blood, and its effect is markedly reduced in the presence of fibrin. Spontaneously lytic blood contains varying amounts of activator bound by antiactivator, but no or little plasmin bound by antiplasmin. These observations indicate that activation of plasminogen takes place on the fibrin surface, the effect of the inhibitor against activator being reduced by adsorption of this enzyme to fibrin.

Recent studies on the properties of the activator and proactivator have shown that the activator activity deteriorates rapidly at acid and alkaline reactions. The proactivator is very stable, especially at acid reactions. The effect of the activator is increased markedly by di- and tricarboxylic acids, while a strong inhibition is produced by diamino-monocarboxylic acids and lysine ethyl ester.

Plasminogen and plasmin.

The existence of the proactivator-activator system in blood is of significance also for an evaluation of the properties of different preparations of plasminogen and plasmin. All preparations of human plasminogen contain proactivator and a complete activation is produced by addition of streptokinase. Animal plasmin has been prepared from plasminogen by spontaneous activation or by a treatment with chloroform or tissue activator. A complete activation is not obtained by these means, but has been accomplished by means of human activator. Preparations of plasmin may be freed from activators and inhibitors by a heat treatment at acid reaction.

Plasmin is a protease, which splits a number of proteins. It has a pH of optimum activity about 7.8. It is very stable at acid reactions, losing only 60-70% of its activity after 30 minutes at pH 1.8 and 100°C. The stability at neutrality is high in crude and rather low in purified preparations and the deterioration is probably mainly due to autodigestion.

Mechanism of fibrinolysis in blood.

A hypothesis for the mechanism of activation and effect of plasmin is set forth.

SAMMENDRAG

Plasminogenets aktivering.

Plasmin er blodets proteolytiske og fibrinolytiske enzym. Det forekommer normalt i blodet som et inaktivt forstadium, plasminogen. I den levende humane organisme øges blodets fibrinolytiske aktivitet ved tilstande af emotionel og fysisk belastning og i udtalte tilfælde udvikles afibrinogenæmi, ukoagulabelt blod og hæmorrhagisk diathese. Efter døden udvikles ofte en betydelig fibrinolytisk aktivitet i blodet. En øget karpermeabilitet og udsivning af vævskomponenter i blodet synes at være vigtige faktorer for udviklingen af fibrinolyse i organismen. In vitro kan en omdannelse af plasminogen til plasmin fremkaldes ved bestemte fældningsmetoder eller ved en behandling af serum eller globulinfraktioner med chloroform, væv eller trypsin. Filtratet fra kulturer af visse streptokok- og staphylokokstammer indeholder stofferne, streptokinase og staphylokinase, der kan fremkalde aktivering af plasminogen. Ved udfældning af globulinfraktionen og ved chloroformbehandling fjærnes eller ødelægges inhibitorer i serum. Det er vist, at vævsaktivatoren reagerer støkiometrisk med plasminogen i en ligevægtsproces. Det er nu sikkert, at plasmin ikke aktiverer plasminogen, medens trypsin er i stand hertil. Streptokinase omdanner humant plasminogen til plasmin, men ikke eller kun i ringe grad plasminogen fra dyr. Denne og en række andre iagttagelser har tydet på, at aktiveringen af plasminogenet var mere kompliceret end hidtil antaget.

Plasminogenaktivatoren i blod.

Nyere undersøgelser over streptokinasens virkning på humant og bovint plasminogen har vist, at streptokinase ikke virker direkte på plasminogen, men indirekte over et proaktivator-aktivator system. Videre undersøgelser over reaktionsmåden har vist, at streptokinase reagerer med proaktivator i en reversibel ligevægt:

Streptokinase + proaktivator $\rightleftarrows$ aktivator + inaktiv komponent.

Det er muligt, at streptokinase reagerer med endnu en komponent i blod inden dannelsen af aktivator fra proaktivator finder sted. Undersøgelser over aktivatorens virkning på bovint, proaktivatorfrit, plasminogen har vist, at aktivatoren er et proteolytisk enzym, der katalyserer omdannelsen af plasminogen til plasmin:

$$\text{plasminogen} \xrightarrow{\text{aktivator (plasminogenase)}} \text{plasmin}$$

Den spontane dannelse af plasmin in vivo og in vitro sker sandsynligvis også over et proaktivator-aktivator system. En kraftig plasminogenaktivator forekommer i spontant lytisk humant blod, og det er lykkedes at påvise en faktor med en lignende virkning som streptokinase i disse blodprøver og i visse vævsekstrakter.

Forekomsten af en proaktivator foruden plasminogen i humant blod støttes af stabilitetsundersøgelser over forstadierne til lysinethylester esterasen og tosylargininmethylester esterasen i humant blod.

Disse iagttagelser muliggør en forklaring af en række tidligere uoverensstemmelser. Den manglende effekt af streptokinase på bovine plasminogenpræparater skyldes således mangel på proaktivator. Humane præparater indeholder store mængder proaktivator, og streptokinaseaktiverede og spontant lytiske præparater fra humant blod indeholder betydelige mængder aktivator foruden plasmin. Den udtalte lytiske effekt af disse præparater på det almindeligt benyttede bovine fibrin substrat skyldes praktisk talt udelukkende aktiveringen af det plasminogen, der findes i dette substrat. De fleste tidligere undersøgelser over fibrinolytisk aktivitet af blod fra levende og døde mennesker og over streptokinase-aktiverede præparater fra humant blod har derfor fortrinsvis drejet sig om aktivatoren og ikke om plasmin.

Eksistensen af proaktivator-aktivator systemet har også betydning for opfattelsen af udviklingen af fibrinolyse i blod. I rensede præparater sker den af plasmin og aktivator forårsagede spaltning af fibrinogen og fibrin med omtrent samme hastighed. I plasma, i nærværelse af inhibitorer, spaltes kun fibrin, men ikke fibrinogen. Aktivator adsorberes stærkt til fibrin. Der findes en antiaktivator i blod og dets virkning mindskes stærkt i nærværelse af fibrin. Spontant lytisk blod indeholder forskellige mængder aktivator bundet af antiaktivator, men intet eller lidet plasmin bundet af antiplasmin. Disse iagttagelser tyder på, at plasminogenets aktivering sker på fibrinets overflade, idet virkningen af inhibitoren mod aktivator mindskes ved adsorbtionen af dette enzym til fibrin.

Nyere undersøgelser over aktivatorens og proaktivatorens egenska-

ber har vist, at aktivatoraktiviteten hurtigt går tabt ved sur og alkalisk reaktion, medens proaktivatoren er meget stabil især ved sur reaktion. Aktivatorens virkning på plasminogen øges udtalt af di- og trikarboxylsyrer, medens den hæmmes stærkt af diaminomonokarboxylsyrer og af lysinethylester.

Plasminogen og plasmin.

Eksistensen af et proaktivator-aktivator system i blod har også betydning for vurderingen af egenskaberne af forskellige præparationer af plasminogen og plasmin. Alle præparater af humant plasminogen indeholder proaktivator og en fuldstændig aktivering opnås ved tilsætning af streptokinase. Animalsk plasmin er blevet fremstillet fra plasminogen ved spontan aktivering eller ved behandling med chloroform eller vævsaktivator. En fuldstændig aktivering opnås ikke herved, men kan opnås med human plasminogenaktivator. Plasmin kan renses for aktivatorer og inhibitorer ved en varmebehandling ved sur reaktion.

Plasmin er en protease, som spalter en række proteiner. Det har et aktivitetsoptimum omkring pH 7.8. Det er meget stabilt ved sur reaktion, idet kun 60-70% af aktiviteten tabes efter 30 minutters opvarmning ved pH 1.8 og 100°. Ved neutral reaktion er det meget stabilt i urene præparater, men labilt i rensede præparater, og ødelæggelsen skyldes formentlig hovedsagelig selvfordøjelse.

Fibrinolysens mekanisme i blodet.

Der fremsættes en hypotese over plasminets aktiverings- og virkningsmekanisme i blod.

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